IODINE DEFICIENCY IN A COMMONLY USED STOCK DIET

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It was shown in previous work by the authors ('35) that the addition of 1% cod liver oil to a supposedly complete diet produced thyroid glands in female rats which were significantly smaller than those of their litter-mate controls. Since it is known that the thyroid gland is sensitive to iodine, the possibility that the iodine present in cod liver oil was responsible for the changes suggested itself. Dr. Roe E. Remington, in a personal communication, suggested, as a result of his observations, that the changes might possibly be due to the addition of iodine (in cod liver oil) to an iodine deficient diet.

The present work was carried on to find out whether the change could be found in males; whether time had any influence upon the change; and whether the change was due to the iodine in the administered cod liver oil.

MATERIALS AND METHODS

Male Wistar albino rats were used. The animals were weaned at 3 weeks of age and immediately placed on the diets. The basal diet consisted of: casein, 15%; whole milk powder, 10%; sodium chloride, 0.8%; calcium carbonate, 1.5%; butter (unsalted), 5.2%; and whole ground wheat, 67.5%. The mixture was prepared fresh at least every other day.

The animals were divided into three series. Series A consisted of twenty-five males which received the basal diet plus

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2% pure cod liver oil (U.S.P.). Twenty-five litter mates received only the basal diet. The animals of this group remained on the diet until 4 months of age and were then autopsied.

In series B twenty-six males received the basal diet plus 2% cod liver oil. Twenty-six litter mates were sustained on the basal diet. The animals of this group were kept on the diet until the age of 3 months and then killed.

Series C consisted of five groups of male rats. Twenty-five were sustained on the basal diet; twenty-six had 1% oil added to their diet; twenty-six received 2% oil; twenty-six received 0.0184% potassium iodide, and twenty-six animals received 0.0184% potassium iodide plus 2% cod liver oil in addition to the basal diet described above. The rats of this series were autopsied at 2 months of age. The source of the iodine was Morton's iodized salt which the manufacturers state contains 0.023% of potassium iodide.

The progress of each animal was carefully watched, and each was weighed weekly. At the time of autopsy in the 4-month and 3-month series, the body and tail lengths were measured in addition to the weights of the head, suprarenal glands, hypophysis, thyroid, thymus and testes. In the 2-month series the lengths of the body and tail were measured as well as the weights of the head and the thyroid gland. The organs removed were fixed in Bouin's fluid. Sections of the thyroid glands of the 2-month series were stained with hematoxylin and eosin.

A comparison of the results from the various groups of animals was made, using modern biometrical methods. As is customary the difference between the two means divided by the probable error of the difference is spoken of as the significance ratio in this paper.

OBSERVATIONS

Thyroid gland

Series A. The mean thyroid weight (table 1) of the rats without oil in their diet was 0.02620 gm. The average weight

of the thyroid of those animals which received oil was 0.02025 gm. The 22.71% decrease in the oil group was definitely significant. The significance ratio for the difference was 7.88.

Series B. The thyroid gland of the animals in this group (table 2) showed a definite weight change. The mean value

TABLE 1

Statistical summary of results of the 4-month group.
(Measurements in grams and centimeters)

	MEAN		DIFFERENCE BETWEEN MEANS	SIGNIFICANCE RATIO
	Oil	No oil		
Hypophysis	0.00868±0.00015	0.00838±0.00012	0.00030±0.00019	1.59
Thyroid	0.02025±0.00044	0.02620±0.00061	0.00595±0.00075	7.88
Thymus	0.53233±0.01790	0.52694±0.01819	0.00539±0.02552	0.21
Suprarenals	0.02827±0.00051	0.02742±0.00045	0.00085±0.00068	1.25
Testes	2.9447 ±0.0494	3.1237 ±0.0462	0.1790 ±0.0676	2.65
Body weight	300.8 ±5.2	290.8 ±5.1	10.04 ±7.28	1.38
Head weight	23.08 ±0.30	22.50 ±0.25	0.58 ±0.39	1.47
Body length	22.32 ±0.11	22.18 ±0.11	0.14 ±0.15	0.91
Tail length	20.68 ±0.15	20.51 ±0.17	0.16 ±0.23	0.71

TABLE 2

Statistical summary of results of the 3-month group.
(Measurements in grams and centimeters)

	MEAN		DIFFERENCE BETWEEN MEANS	SIGNIFICANCE RATIO
	Oil	No oil		
Hypophysis	0.00824±0.00012	0.00826±0.00015	0.00002±0.00019	0.10
Thyroid	0.02010±0.00053	0.02596±0.00052	0.00586±0.00074	7.93
Thymus	0.6225 ±0.01738	0.68070±0.01829	0.05820±0.02523	2.31
Suprarenals	0.02718±0.00036	0.02784±0.00050	0.00065±0.00062	1.06
Testes	2.99259±0.03221	3.15876±0.06913	0.16617±0.07626	2.18
Body weight	274.2 ±3.7698	273.0 ±4.7011	1.2 ±6.026	0.20
Head weight	21.89 ±0.1970	21.69 ±0.2835	0.20 ±0.3452	0.58
Body length	22.08 ±0.1140	21.81 ±0.1219	0.27 ±0.1669	1.62
Tail length	20.39 ±0.1016	20.29 ±0.1231	0.10 ±0.1596	0.63

for the no-oil animals was 0.02596 gm. while that for the rats with 2% oil was 0.02010 gm. The decrease in the oil group amounted to 22.57%. The difference was significant, the significance ratio being 7.93.

Series C. The mean autopsy weight (table 3) of the several groups of this series were: no-oil, 0.01646 gm.; KI-no oil,

0.01271 gm.; 1% oil, 0.01464 gm.; 2% oil, 0.01427 gm.; and KI-2% oil, 0.01262 gm. The no-oil glands were larger than the other glands. The KI-no oil glands were decreased 22.80% when compared to the no-oil group; the 1% oil group, 11.04%; the 2% oil group, 13.31%; and the KI-2% oil group, 23.31%. All of these decreases were significant as shown by the following significance ratios: KI-no oil, 7.46; 1% oil, 3.51; 2% oil, 4.14; and KI-2% oil, 8.24 (no-oil group used for comparison).

TABLE 3

Means of 2-month group. (Measurements in grams and centimeters)

	NO OIL	KI-NO OIL	1% OIL	2% OIL	KI-2% OIL
Thyroid	0.01646 ±0.00038	0.01271 ±0.00033	0.01464 ±0.00035	0.01427 ±0.00037	0.01262 ±0.00027
Body weight	173.48±3.37	183.92±4.11	186.42±4.07	185.62±3.42	179.38±4.18
Head weight	15.90±0.23	16.26±0.27	16.26±0.26	16.24±0.25	15.90±0.28
Body length	19.29±0.11	19.50±0.12	19.48±0.13	19.43±0.11	19.27±0.14
Tail length	16.86±0.12	17.30±0.14	17.37±0.14	17.11±0.12	17.13±0.14

TABLE 4

Significance ratios of 2-month group. (No oil compared with other groups)

	KI-NO OIL	1% OIL	2% OIL	KI-2% OIL
Thyroid	7.46	3.51	4.14	8.24
Body weight	1.96	2.45	2.53	1.10
Head weight	1.01	1.04	1.01	0.01
Body length	1.27	1.14	0.90	0.13
Tail length	2.40	2.74	1.49	1.48

The histological structure of the thyroid was studied in six animals of each group. No changes were noted.

Other measurements

The average measurements of body weight, head weight, body length, tail length, hypophysis, thymus, suprarenal glands and testes showed no differences between the tests and controls which were of certain significance in any of the three series. The average measurements are given in the tables and since the results are negative no further discussion is needed.

INCIDENCE OF INFECTIONS

All animals were examined carefully for signs of infections. Very few pathological changes were noted. There was no observed difference in the amount of respiratory or middle ear infection in any of the series studied.

DISCUSSION

Strikingly apparent was the effect of adding cod liver oil to the basal diet used in the present experiment. The thyroid glands of male rats, sustained on diets to which 1% cod liver oil and 2% cod liver oil had been added, at 4 months, 3 months, and 2 months of age were significantly smaller than those of animals without oil in the diet. The same phenomenon has been observed in the case of female rats at 4 months of age (Freudenberger and Clausen, '35). Interesting enough was the fact that no significant changes were found in any of the other structures studied in the 4-month and 3-month series. On this basis the gonads, hypophysis, thymus, and suprarenal glands were not studied in the 2-month series.

Naturally, the question arose as to which constituent, or constituents, of the cod liver oil was responsible for these definite and consistent weight changes in the thyroid gland with no significant alteration in any of the other structures studied. The adequacy of the diet together with the close parallelism of the various groups of animals both as to body growth and weights of the several organs (except thyroid), most certainly spoke against the possibility of any form of an avitaminosis.

The sensitivity of the thyroid to the iodine intake is an established fact. This seemed to be by far the most likely constituent of the cod liver oil responsible for the changes. The effects of iodine on the thyroid gland have been studied by many investigators. Hayden, Wenner and Rucker ('23–'24) found thyroid enlargement in rats on a diet containing a small amount of iodine (9 to 10 parts per billion). In the same period McClendon and Williams made similar observations. By the addition of iodine, these authors found that

smaller thyroid glands were obtained. Marine ('24) maintained that simple goiter was caused by a deficiency of iodine. Tanabe ('25) confirmed Marine with results obtained from the rat. Krause and Monroe ('30) found that the addition of iodine to diets low in this constituent resulted in smaller thyroid glands in rats. Thompson ('32) reported enlargement of thyroids in rats on a rachitogenic diet. The addition of iodine, not vitamin D, produced smaller glands. Levine, Remington and von Kolnitz ('33) worked out a range of iodine dosage which was proved to be very sensitive. The range was between 0.14 γ and 0.59 γ ($\gamma = 0.001$ mg.) daily. Small variations in the amount of iodine within this range produced marked changes in the thyroid glands. The authors came to the conclusion that the minimum daily intake of iodine to prevent goiter in the rat was from 1 to 2 γ.

Cod liver oil as a source of iodine is without dispute. Holmes and Remington ('35), reporting on the iodine content of twenty representative samples of American cod liver oil, found the iodine content to average 8640 parts per billion parts of cod liver oil.

The evidence, from the data obtained with the 4-month and 3-month series, was very suggestive that the basal diet fed the animals was inadequate in iodine. That is, the rats received iodine which perhaps fell within the critical range of iodine intake mentioned above (less than $\frac{1}{2}$ γ daily).

In the 2-month group iodine in the form of potassium iodide was added to the basal diet to find out whether the glands could be reduced in size, as was the case with the administration of cod liver oil. The iodized salt was used in place of the normal salt in the basal diet. The amount of potassium iodide in the salt was stated as 0.023%. Since the amount of salt in the basal diet was 0.8%, the amount of potassium iodide was calculated to be 0.0184%. This value would give each animal approximately 14 γ of iodine daily (assuming each animal ate 10 gm. of food daily). Such an iodine intake is far in excess of the physiological requirement for the rat.

The results showed that the administration of potassium iodide to the basal diet produced a much greater change in the weight of the thyroid gland than was found with the administration of either 1 or 2% of cod liver oil. It would seem, therefore, that the iodine in the cod liver oil was not available to the organism in sufficient quantities to render the basal diet completely antigoitrogenic (did not remove the iodine intake from the critical range cited above). Thus, iodine in the form of potassium iodide was more effective in preventing goiter than the iodine in the cod liver oil.

An unexplainable bit of information was the fact that the 2% oil had practically no more effect than did the 1% oil. Certainly, one would think that if 1% cod liver oil was not sufficient to bring the intake of iodine out of the critical range, 2% oil would produce a change greater than that produced by the 1% oil. Such was not the case. Two per cent cod liver oil did not have the magnitude of influence in the 2-month series that it did in the 3- and 4-month series. This fact may or may not have significance.

Remington, Remington and Welch ('37) reported normal thyroid weights which were lower than those reported in the literature with the exception of those of McCarrison ('30). They called attention to the importance of an adequate amount of iodine in the diet and suggested that a stock diet such as that used by us was probably deficient in iodine. Our results would tend to confirm their contentions.

SUMMARY AND CONCLUSIONS

A total of 231 male Wistar albino rats was divided into three series. The animals of all series were placed on the various diets at 3 weeks of age. Series A consisted of twenty-five rats fed a well-balanced basal diet plus 2% cod liver oil, and twenty-five rats fed on the basal diet for controls. The animals were killed and autopsied at 4 months of age. Series B was composed of twenty-six test rats and twenty-six controls treated exactly as those of series A, except that the animals were killed and autopsied at 3 months of age. In series

C there were five groups of rats. Twenty-five were sustained on the basal diet; twenty-six had 1% oil added to the diet; twenty-six received 2% oil; twenty-six had 0.0184% potassium iodide added to the basal diet; and twenty-six animals received the same amount of potassium iodide plus 2% cod liver oil. The rats of this group were autopsied at 2 months of age.

No significant difference in any of the series was found in body weight, body length, tail length, incidence of infection, or in the weights of the head, suprarenal glands, hypophysis, thymus, or testes.

The thyroid gland was significantly smaller in all groups of animals which received either cod liver oil or potassium iodide, or both. The change was most marked in those animals which had potassium iodide present in their diets.

The conclusion was reached that the stock diet fed the animals of this colony is iodine deficient. This condition was compensated for in part by the addition of cod liver oil. Potassium iodide added to the stock diet was more efficacious in obviating the condition. There was apparently no difference between the sexes. There seemed to be a greater change in thyroid weight in the series of animals sustained on the diets for the longer periods of time, namely, 3 and 4 months.

Lastly, iodized salt should be substituted for ordinary salt in basal diets, at least in goitrogenic belts, in order to supply the thyroid gland (and the organism) with the physiological requirement of iodine. The result will be a lower normal value for thyroid gland weights.

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VITAMIN G AND SYNTHETIC RIBOFLAVIN [1]

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ONE FIGURE

(Received for publication July 14, 1937)

Vitamin G (B_2) previous to 1933 was defined (American Society of Biological Chemists, '29) as the heat-stable water soluble dietary factor necessary for maintenance and growth in the rat. It was generally assumed the same factor prevented the occurrence of a dermatitis which frequently accompanied this deficiency (Goldberger and Lillie, '26; Sherman and Sandel, '31). The identity of vitamin G to the P-P factor of Goldberger was not clear, but the facts at that time made it seem probable that they were the same (Goldberger, Wheeler, Lillie and Rogers, '26; Sherman and Smith, '31). The evidence now indicates that several factors were involved in this deficiency as previously defined (Gyorgy, '35; Harris, '35; Koehn and Elvehjem, '36). It is therefore important to be able to interpret the earlier rat tests in terms of newer knowledge.

The Sherman-Bourquin diet ('31) which contains an alcoholic extract of ground whole wheat as a source of vitamin B_1 and other possible essentials in this group was very widely used for vitamin G determinations. It is the object of this paper to show that this method was a test for riboflavin and to report the rat growth response to synthetic riboflavin of known purity.

[1] Aided by a grant from the Milton Fund to the University Committee on Research in Dental Medicine.

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Using a diet in which a yeast preparation supplied the water-soluble accessory factors other than riboflavin Karrer ('35) found 5.5 gm. weekly growth when 3 γ daily of riboflavin were fed. On a similar diet Euler and others ('34) reports 7.7 gm. weekly response to 5 γ daily and Kuhn ('35) obtained 10.2 gm. growth with 10 γ daily.

Kuhn ('34) also reported that it was necessary to add a 'B_4 concentrate' to the Bourquin diet in order to obtain a growth response to riboflavin. On the contrary, several papers (Bisby and Sherman, '35; Ansbacher, Supplee and Bender, '36; Copping, '36) have indicated that this method probably was an assay of riboflavin. However, the evidence has not been conclusive because the purity of the riboflavin preparations was not established. The potency of preparations from different laboratories vary considerably, no doubt because many of them to which the term lactoflavin (riboflavin) has been applied are only concentrates. To obtain a yellow powder or a crystalline material is no criterion of purity. Experience has shown that such preparations may be far from pure compounds.

The riboflavin used in these experiments was synthesized by a method similar to that used by Karrer ('35). It is a bright orange yellow substance which easily crystallizes from water and although the substance after the first recrystallization was homogeneous, it was necessary to recrystallize it many times before a constant melting point was reached. The pure substance has a melting point of 293°C.[2] Nitrogen 14.80 (calculated 14.88). The acetyl derivative was prepared (M.P. 239°C.) and reconverted to ribofiavin, which had the same properties as the original.

The tests were carried out in every detail as previously described (Bourquin and Sherman, '31). Rats were transferred from a wheat and milk diet at 28 days (45 to 50 gm.) to a basal diet composed of purified casein, 18 parts, salt mixture

[2] The substance slowly decomposes above 275° and therefore the melting point by the usual method is not a good constant. By the use of a block, the instantaneous melting point can serve as a useful criteria of purity.

4 parts, butter fat 8 parts, cod liver oil 2 parts, and cornstarch 68 parts, a part of which carried the alcoholic extract of 500 gm. of whole wheat (hard, red) for each kilo of diet.

The rats showed slight weight gains for about 2 weeks, after which time their weights became constant. The supplement of pure riboflavin was started on the fifth week. An accurately

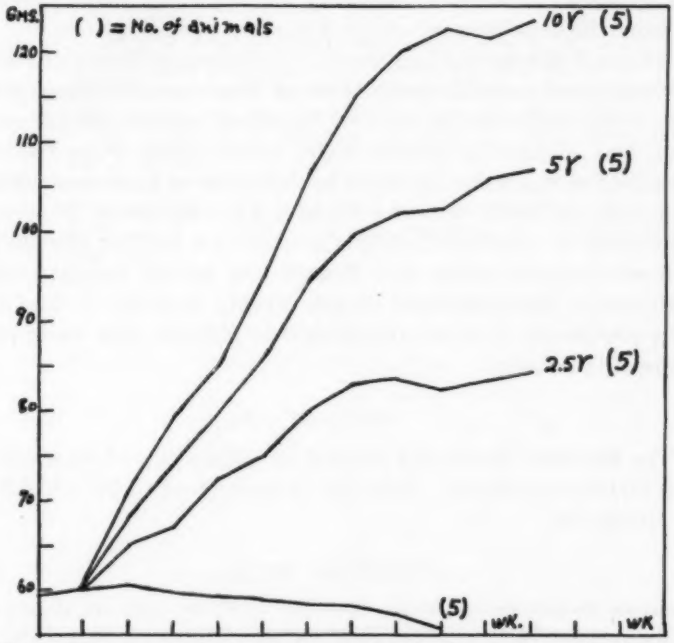

Fig. 1 The average growth of rats receiving the Sherman-Bourquin vitamin G diet plus variable amounts of pure synthetic riboflavin.

prepared solution was fed six times weekly by means of a small graduated pipette. This substance is bitter and it is therefore necessary to be certain that the complete dose is consumed.

Weight response was prompt, uniform and nearly proportional to the levels fed. After 6 weeks the weights of the animals on the lower level remained about constant. A similar

decrease in growth rate occurred on the higher levels a week or so later. At least a part of this is due to the larger size of the animal because an increased supplement of riboflavin will result in resumption of growth. However, the ratio of weight gain to riboflavin beyond this period indicates that probably some other dietary essential is becoming the limiting factor for growth. Such animals may be kept for many weeks without the development of any apparent pathology.

Although the weight increments for increased levels of riboflavin are not so uniform as those of Sherman and Bourquin, this is no doubt due in part to the small number of animals used and in part to growth rates larger than those recommended for this test. It would be desirable to have more data at riboflavin levels between 2 γ and 4 γ. However, it seems reasonable to conclude that this method is a test for riboflavin and calculations based on a 6-week test period indicate that a Sherman Bourquin unit (3 gm. weekly growth) is 2.0 γ to 2.5 γ riboflavin, or there are 400,000 to 500,000 such units per gram of riboflavin.

SUMMARY

The Sherman-Bourquin method of estimation of vitamin G is a test for riboflavin. One unit is equivalent to 2.0 γ to 2.5 γ of riboflavin.

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EFFECT OF DIETS CONTAINING FATS OF VARIOUS DEGREES OF UNSATURATION ON THE SERUM LIPIDS IN RATS[1]

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The investigations of various observers (Henriques and Hansen, '01, '02; Ellis and Isbell, '26; Anderson and Mendel, '28) indicate that the character of depot fat is dependent upon the nature of the diet. The relationship of the diet to blood lipids, on the other hand, has been studied relatively little. Recent reports by Hansen and Burr ('33), Williams and Maynard ('34), Hansen, Wilson and Williams ('36), Hansen and Brown ('37), Brown, Hansen, McQuarrie and Burr ('37) indicate that the blood lipids tend to be less unsaturated when the animal organism is restricted to a low fat regimen. Hansen ('33) first observed the effect of dietary fat on serum lipids when he obtained an increase in the iodine number of the serum fatty acids of certain eczematous individuals following the administration of linseed and corn oil. Similarly Hansen, Wilson and Williams ('36) working with dogs found the iodine numbers of the isolated fatty acids of the serum lipids varied in the same direction as the iodine number of the dietary fats. This is a report of an earlier study in which rats were fed a group of common commercial fats possessing a wide range of iodine numbers.

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The same plan of study and chemical methods were used as in the previous study of this series (Hansen and Brown, '37). Fats were fed at 20% levels, coconut oil, olive oil, lard, Crisco (hydrogenated cottonseed oil), corn oil and linseed oil being used. The average iodine numbers of the various dietary fats as determined are found in table 2. Four or five individual animals were used for each type of food fat. The serum lipids were determined on two or three individual animals of each group and in addition pooled samples were made from two to three animals on the same type of diet. In the large samples it was possible to make duplicate determinations throughout.

The results of the study are presented in table 1.

DISCUSSION

Although the number of animals in each case is few, there is a surprising uniformity in the values observed. The results obtained in the cases of pooled samples of blood from several animals where all the determinations were done in duplicate are in close agreement with that of the average of the group. The iodine numbers of the serum lipids are highest in the groups fed on linseed and corn oil, which substances have the greatest degree of unsaturation. The intermediate values as found in the groups fed on lard and Crisco are consistent with the lesser degree of unsaturation of these substances. The low value for the coconut oil group is in keeping with the saturated condition of this fat. The values obtained for the iodine numbers of the serum lipids for the animals fed the olive oil are rather unexpected in view of the fact that the leaf fat-fatty acids possessed an iodine number of 81 while those of the lard and Crisco rats were 73 and 70, respectively.

As shown in table 1 there is an inverse relationship between the level of the total lipids and their degree of unsaturation. The rats fed on the linseed oil diet have small quantities of total lipids while the coconut oil animals have large amounts of total lipids. The most marked difference is found in the values of the total fatty acids, the same general relationship holding as for the total lipids. The cholesterol values tend

TABLE 1

Serum lipids of rats fed on diets containing fats of various degrees of unsaturation

DIETARY FAT	COLONY NO.	IODINE ABSORBED, MG./100 CC.	CHOLESTEROL, MG. %	TOTAL FATTY ACIDS, MG. %	TOTAL LIPIDS, MG. %	IODINE NUMBERS OF TOTAL LIPIDS
Linseed oil	489, 492, 6	463	72	264	336	138
	494	461	103	221	324	142
	490	485	86	279	365	133
	478	332	63	177	240	137
	Average	435	81	235.2	316.2	137.5
Corn oil	476, 493, 498	519	89	306	395	132
	499	518	113	254	367	141
	497	506	102	302	404	125
	477	442	75	253	328	135
	Average	496.2	94.8	279	373.5	135
Lard	21, 15, 17	588	81	382	463	127
	18	518	105	274	379	136
	19	614	95	437	532	115
	2	549	92	357	447	123
	Average	567	93.2	362.5	455.2	125
Crisco	3, 4, 5	547	81	395	476	115
	479	508	99	254	353	144
	1	548	109	351	460	119
	Average	534.3	96.3	333.3	430	126
Olive oil	462, 467	495	88	342	430	115
	468	503	97	336	433	116
	466	630	97	386	483	130
	Average	542.7	94	355	449	120
Coconut oil	5, 6	513	106	352	457	112
	11	582	119	380	499	116
	4	573	112	367	479	119
	3	490	89	434	523	94
	Average	539.5	106.5	383.2	490	110

to remain more constant, although they are also lowest in the linseed group and highest in the coconut group. The exact significance of this inverse relationship is not evident; however, it suggests the possibility that the fatty acids of the serum tend to maintain a rather uniform composition as to the number of unsaturated bonds present.

The degree of unsaturation of the serum lipids is definitely less in animals reared on the low fat ration (Hansen and Brown, '37) than in rats given fat in their diets. The average iodine number of the depot fat of rats maintained on the low fat regimen was found to be 66, whereas in the animals fed coconut oil, the average iodine number of the body fat was 54.

TABLE 2

Average iodine numbers of the various dietary fats as compared with the depot fat and the serum lipids of rats

TYPE OF FAT	AVERAGE IODINE NUMBER		SERUM LIPIDS
	Dietary	Depot	
Linseed oil	160	118	138
Corn oil	118	107	135
Olive oil	85	81	120
Lard	67	73	125
Crisco	69	70	126
Coconut oil	13	54	110

There is a distinct reversal in the average iodine number of the serum lipids. In the group fed on the low fat diet the total lipids of the serum had an average iodine number of 90, while in the cases of the animals reared on coconut oil the iodine number was 110. Thus it appears that a regimen low in fat depresses the degree of unsaturation of the serum lipids more than a diet fairly high in fat but containing mostly saturated fatty acids. This observation, coupled with that of the differences found between the olive oil and lard and Crisco reared animals, suggests the probability that the unsaturated acids are selectively retained by the blood. In general the results are considered to support the view that the rat depends upon the diet for its unsaturated fatty acid supply. The fact that the blood picture can be changed so readily by dietary

means indicates the importance of regulating this factor in metabolic studies which involve the serum lipids. Our findings show that the same general relationship exists between the dietary fats and blood lipids as was found by Anderson and Mendel ('28) to exist between dietary fats and body fats.

SUMMARY AND CONCLUSIONS

1. When rats were reared on diets containing fats of various degrees of unsaturation the iodine numbers of the total lipids of the serum tended to vary directly with the iodine number of the dietary fats.

2. The values for the total lipids were found to vary inversely with the degree of unsaturation of the serum lipids.

3. The results indicate the possibility of a selective retention of the unsaturated fatty acids of the blood.

4. These data indicate that the character of the diet must be taken into consideration in the interpretation of metabolic studies involving the serum lipids, particularly as regards the degree of unsaturation of the fatty acids.

5. The results show that a positive correlation exists between the degree of unsaturation of food fat, depot fat and the blood fat.

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BASAL METABOLISM OF OKLAHOMA MEN AND CHILDREN

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ONE FIGURE

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For several years it has been doubted whether the northern standards of basal metabolism (DuBois or Benedict) can be generally used satisfactorily, and experimental evidence has actually shown these values for women to be too high for use in the south. Hafkesbring and Borgstrom ('26) reported basal metabolic rates for normal women in New Orleans that were below the northern standards. Tilt ('30) from a study of Florida women reported similar results. Coons ('31) in this laboratory from a study of 101 Oklahoma college women reported an average basal metabolic rate of —13.2%. The question has arisen as to whether Oklahoma men and children show a correspondingly low basal metabolism. Therefore, in order to make the study complete, data were compiled on the basal metabolic rate of eighty-four children of both sexes and seventy-five men from this State.

PROCEDURE

The Benedict-Roth recording metabolism apparatus was used for all tests. The usual precaution in testing each time for leaks and often for efficiency of the soda lime for CO_2 and H_2O absorption was observed. All tests were run early in the morning, the subject being in the post absorptive state and having rested 30 minutes just previous to the determination.

BASAL METABOLISM OF OKLAHOMA MEN

The seventy-five men whose metabolic rates were studied were chosen indiscriminately from men enrolled in this college. They were all normal, healthy young men, many of whom were in athletic training, and varying in age from 17 to 30 years; the majority falling within the range from 18 to 24. The data so obtained are summarized in table 1.

The data from these subjects were assembled into age groups as indicated for convenience in handling and because the number of subjects of each age studied was not sufficiently large to merit separate consideration.

An inspection of table 1 indicates the lowered basal metabolic rate shown by Oklahoma men. The average rate for all

TABLE 1

AGE	NUMBER	AVERAGE WEIGHT (KG.)	AVERAGE SURFACE AREA (M^2)	AVERAGE TOTAL HEAT PRODUCTION (CAL./24 HOURS)	AVERAGE (CAL./M^2 PER HOUR)	AVERAGE B.M.R. (%)
17–19	27	65.75	1.79	1762.9	41.03	—4.42
20–24	39	72.14	1.91	1761.3	38.40	—6.34
25–29	9	72.60	1.87	1696.8	37.80	—6.20

age groups was found to be —5.63%. Of all subjects studied 76.4% fell within ±10% deviation from the DuBois normal; 21.9% fell below —10%; and 8.2% were below —15%.

Figure 1 represents graphically the distribution of the basal metabolic rate results of all the subjects over the range from —24% to +18%. The fact that the mode is in the range from —6% to —12% would lead one to believe that the average basal metabolic rate of the entire group would be somewhere in that range rather than the calculated —5.63%, but the close approximation of the 0 to —6 frequency to that of the mode along with the shewness of the variation curve to the right accounts for this.

BASAL METABOLISM OF OKLAHOMA CHILDREN

The basal metabolic rates of eighty-four children (forty-two boys and forty-two girls) ranging in age from 5 to 15 years

were determined. These children might be referred to as a privileged group having been selected from homes where they had the advantages of a select diet and very favorable surroundings. The parents appeared to be normal, healthy individuals. In every case the child was brought from his bed following a night's sleep, and then allowed to rest for an additional 30 minutes. It should be noted that splendid cooperation was obtained from these children. The data so obtained should be indicative of the normal.

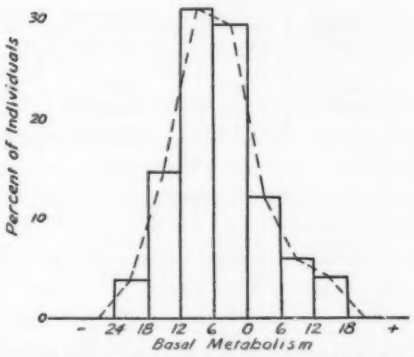

Fig. 1 Frequency polygon and curve showing variation in basal metabolism of seventy-five Oklahoma men.

The results of this group of tests are summarized in two parts: table 2 for girls and table 3 for boys.

The material in the two preceding tables seems to indicate that the basal metabolic rate of the children falls lower from the normal as their age increases, this lowering being most marked with the age group of girls at puberty. The more striking lowering of the basal metabolic rate of young men and young women just beyond these ages is shown by the data of the older boys in the first part of this report. Unpublished data not here represented further substantiate this view for older girls.

The fact that the younger children do not show the low basal metabolic rate of the older ones and of men and women leads one to postulate that they are growing rapidly and since the basal metabolic rate is thought to be a function of the active protoplasmic tissue the rates of these children are as high as those of the north; the deviation from the northern standards coming with the approach of maturity.

TABLE 2

Girls

AGE	NUMBER	AVERAGE WEIGHT (KG.)	AVERAGE SURFACE AREA (M^2)	AVERAGE TOTAL HEAT PRODUCTION (CAL./24 HOURS)	AVERAGE (CAL./M^2 PER HOUR)	AVERAGE B.M.R. (%)
5– 7	20	21.27	0.83	866.85	43.36	+3.36
8–11	16	30.57	1.08	1332.29	51.40	+2.63
12–15	6	42.37	1.37	1713.05	52.10	−6.59

TABLE 3

Boys

AGE	NUMBER	AVERAGE WEIGHT (KG.)	AVERAGE SURFACE AREA (M^2)	AVERAGE TOTAL HEAT PRODUCTION (CAL./24 HOURS)	AVERAGE (CAL./M^2 PER HOUR)	AVERAGE B.M.R. (%)
4– 7	12	21.36	0.83	861.60	43.20	+1.72
8–11	24	31.81	1.12	1457.68	54.23	−2.34
12–14	6	41.86	1.36	2071.01	63.45	−1.12

CONCLUSIONS

1. The basal metabolism of Oklahoma men is lower than the DuBois standards, the average of seventy-five normal men being —5.63% deviation from the DuBois normal.

2. The basal metabolism of Oklahoma children agrees with the northern standards while they are very young but gradually become lowered with increasing age as shown in these child studies and in the results of younger men here reported, and for girls previously reported from this laboratory.

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VITAMIN B_2 DEFICIENCIES AS AFFECTED BY DIETARY CARBOHYDRATE

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FIVE FIGURES

(Received for publication July 6, 1937)

In the course of certain attempts to produce and to prevent cataracts in rats by dietary means there were observed in this laboratory striking differences in the response of rats to basal diets similar in all respects except as to the carbohydrate constituent. Lactose was used in one group of experiments because of the now well-established cataractogenic effect of that sugar (Mitchell and Dodge, '34; Yudkin and Arnold, '35) and vitamin B_2 deficiencies were produced both with this and with the more usually employed cornstarch diet, pursuant to Day, Langston and O'Brien's ('31) finding that cataracts may occur in rats having such deficiencies. It was found at once that while cataracts are produced more or less regularly on lactose-containing diets, the other more usual B_2 deficiency symptoms could not be obtained. This led to the use of sucrose as well as of lactose and cornstarch with varying single and multiple B_2 deficiencies.

The whole problem of the nature, function and even the number of factors included under the designation vitamin B_2, as the British writers have employed the term, is at present too confused for any attempt at review here. Suffice it to say that we have been satisfied by the evidence brought forward by Lepkovsky, Jukes and Krause ('36), Elvehjem and Koehn ('35), Birch, György and Harris ('35), Gorter ('36), and others that at least three distinct factors are involved,

1) riboflavin, 2) an adsorbable heat stable probably basic substance called B_6 by György ('34), and factor 1 by Lepkovsky, Jukes and Krause ('36), 3) one or more unadsorbable factors called filtrate factor by Lepkovsky, Jukes and Krause ('36), B_2 by Elvehjem and Koehn ('35), antiblacktongue or P-P factor by Birch, György and Harris ('35). We have chosen to call these flavin, B_6 and filtrate factors respectively.

The plan involved the use of three similar basal diets with sucrose, lactose or cornstarch as carbohydrate constituent, adequate amounts of the fat-soluble vitamins fed as cod liver oil, crystalline vitamin B_1 (Merck) (10 micrograms per rat per day) as source of vitamin B_1 and one, two or all three of the B_2 factors. There were thus three groups of rats each on the following four regimes: without any of the B_2 factors; with only one, that is flavin or B_6 or filtrate factor; with each of the three possible combinations of two, that is flavin and B_6, flavin and filtrate factor, filtrate factor and B_6; and finally with all three factors. Twenty-seven groups were used, each made up of four to ten animals. Several of the comparisons were repeated three to five times. The growth curves used in this report, however, were taken from concurrent groups carefully chosen from comparable litters since it has become clear that all feeding experiments of this kind must be judged by the parallel performance of littermate controls.

The diet used had the following composition: casein, purified by repeated extractions with hot 95% alcohol and with cold 60% alcohol, 22; Crisco, 9; Osborne and Mendel salt mixture, 4; carbohydrate, 65. In some of the lactose diets a lower proportion of casein was used, 18 or 15%, but no differences in the relative severity of the vitamin deficiencies resulted. The lactose was exposed to strong daylight in thin layers for 7 or 8 days before it was incorporated in the diet and exhibited no fluorescence under 'black light.'

There was little coprophagy among the rats fed these diets. Wide-mesh floor screens on individual cages were used and careful observation made to detect any feces consumption. A small number of suspected animals was removed from the groups.

In most cases the rats, taken at 21 to 28 days of age, were depleted by use of the B_2-free diet for 3 or 4 weeks at which time the weights were found to be stationary or declining except in the case of the lactose groups.

A single level of dosage was chosen for each of the three supplements and only one source of each supplement. Pure crystalline lactoflavin furnished by Dr. S. Lepkovsky and by Vitab Products, Inc. of San Francisco was used at the level of 20 micrograms daily. A series of assays of this material established its high potency, comparable with that reported by Copping ('36), by Ansbacher, Supplee and Bender ('36), and of course, by Lepkovsky and Jukes ('36).

The B_6 preparation was made from autolyzed wheat germ according to the suggestion of Birch and György ('36) and was fed in daily amount equivalent to 0.5 gm. of wheat germ. This extract was tested by Dr. T. H. Jukes on chicks and found to be free from the filtrate factor. Rat assay has confirmed this. The filtrate factor was a rice bran concentrate furnished by the kindness of Vitab Products, Inc. This product was practically free from flavin but contained appreciable amounts of B_6. In all the tests involving the filtrate factor therefore the presence of a small amount of B_6 must be postulated. The standard dose used was 0.5 cc. of the concentrate per rat per day.

The feeding experiments were carried on for varying periods from 12 to 36 weeks, but usually for 14 weeks. Growth, blood and urine sugar, blood and urine calcium, eye lens changes, skin and hair changes were recorded.

THE EFFECT OF LACTOSE

The outstanding findings in the groups on the lactose diet were the complete absence of dermatitis, frequent appearance of cataracts, and subnormal but persistent growth even on the entirely B_2-deficient diet (Morgan and Cook, '36). Addition of either flavin or B_6 or both had little or no effect in improving the growth of these animals. When the filtrate factor was given however either alone or with the other two

factors, a large increase in growth rate resulted and a definite decrease in severity and speed of appearance of the cataracts. With filtrate factor alone added (it being understood that B_1 is present in all cases) the growth achieved as seen in figure 1 is the same as that with 0.5 gm. brewers' yeast (Northwestern) daily. When the flavin and B_6 were also given, the growth is

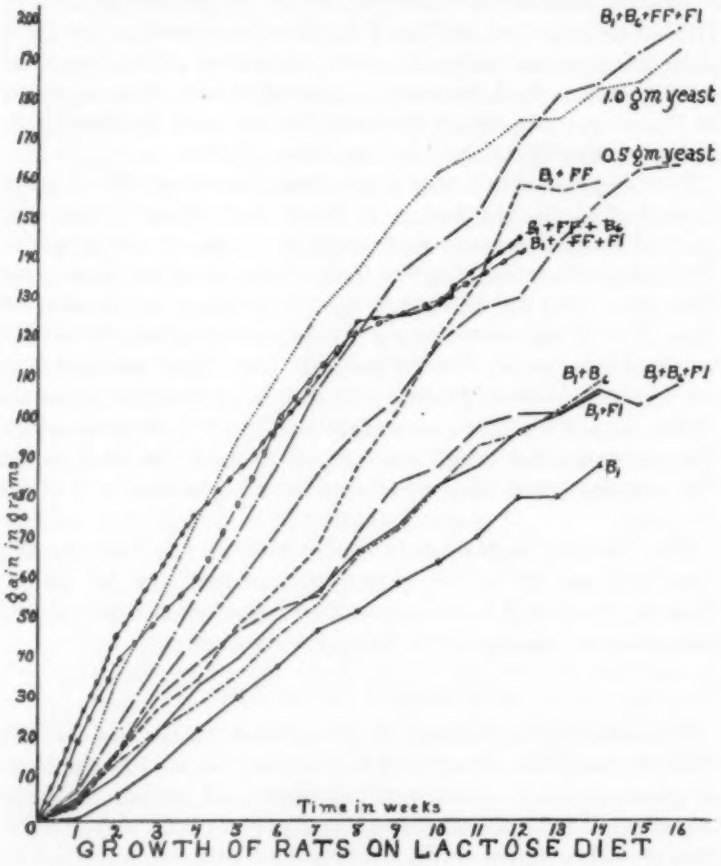

Fig. 1 Growth of young rats on lactose diet with one two, three or all four of the B vitamins: B_1, flavin (Fl), filtrate factors (FF), vitamin B_6 (B_6).

seen to be equal to that promoted by 1.0 gm. daily of the yeast. Apparently 0.5 gm. yeast supplies insufficient amounts of one of the B_2 accessories since 1.0 gm. of yeast is needed to equal the performance of the group given the four factors separately.

All of the lactose-fed rats had much enlarged ceca filled with yellowish white semi-solid contents, apparently mostly unabsorbed lactose. Early in the feeding period some showed diarrhea and a few died during these attacks. Usually after a week or two the diarrhea ceased and the animals appeared to be normal in all respects except for the cataracts and a subnormal rate of growth.

In an earlier experiment groups of rats were fed a similar diet but with 50% lactose and 20% cornstarch instead of 70% lactose and with tiki-tiki as source of all the B vitamins. These had less mature and more delayed cataracts but considerably improved growth when compared with those on the 70% lactose diet. When the proportion of lactose was further reduced to 30%, the cataracts were fewer and less severe but growth was much decreased. These relations are shown in figure 2. Apparently the tiki-tiki was supplemented successfully by the vitamin-forming activity of the lactose at the 50% level, insufficiently at the 30% level and at the 70% level the toxic effect of the lactose became more pronounced than its favorable effect. With cornstarch alone the tiki-tiki allowed very little growth, no cataracts developed but severe dermatitis or hair loss usually occurred. It is probable that the tiki-tiki was lacking chiefly in flavin.

Since B_6 or flavin or both along with the 70% lactose diet brought about little improvement, it would appear that either the lactose contained considerable amounts of these two factors or that they were formed possibly by bacterial activity in the intestine from which adequate quantities were absorbed. Since the addition of the filtrate factor brought about obvious improvement, it must be assumed that the lactose carries none of this and that none is elaborated in the intestine.

In order to determine whether the intestinal contents contain the B_6 and flavin the contents of the ceca of sixty-five lactose-fed rats were washed out with 60% alcohol, filtered, the filtrate concentrated and fed to six rats depleted on the cornstarch diet. These animals resumed growth at a rate

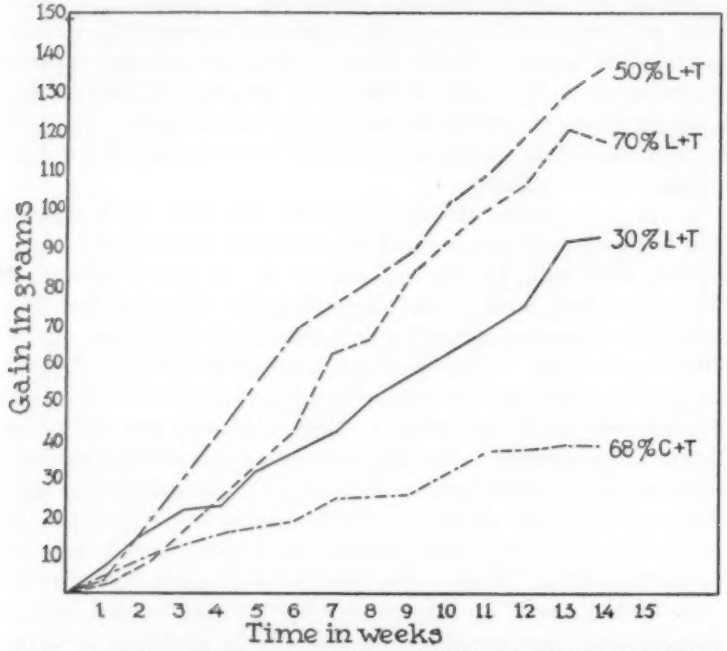

Fig. 2. Growth of young rats on diets containing 30, 50 and 70% lactose (and 40, 20 or 0% cornstarch) and 68% cornstarch with tiki-tiki (rice bran concentrate) as sole source of the B vitamins.

closely paralleling that of littermates on the lactose diet without supplements. The equivalent of the contents of one caecum per rat promoted growth for 3 or 4 days. When the caecum material was no longer given, the growth of these rats continued for 1 week then leveled off as in the negative cornstarch group.[1]

[1] These observations were made by Dorothy V. Rundle.

THE EFFECT OF CORNSTARCH

The rats on the cornstarch diet grew very little without supplements, sometimes developed dermatitis but had no cataracts. See growth curves in figure 3. Their growth was improved by flavin but the dermatitis became more severe and

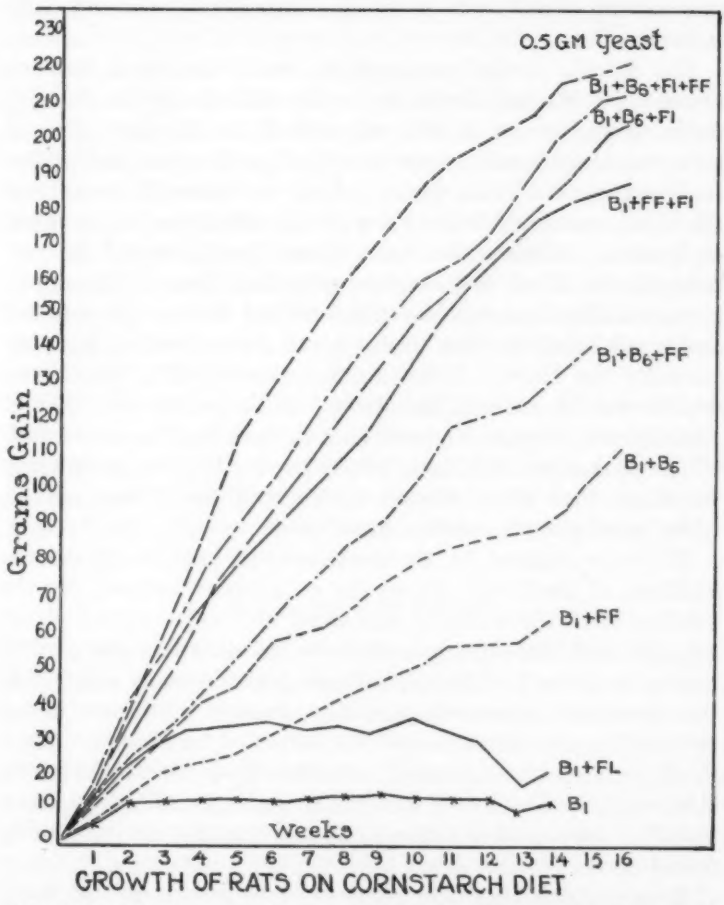

Fig. 3 Growth of young rats on cornstarch diet with one, two, three or all four of the B vitamins: B_1, flavin (Fl), filtrate factors (FF), vitamin B_6 (B_6).

was of the usual 'specific' type with swollen red paws from which the skin was sometimes sloughed off, scabby denuded nose and forelegs and swollen encrusted ears. When B_6 was also given, the dermatitis did not appear and close to normal growth resulted. Addition of the filtrate factor merely increased the growth rate somewhat but alone produced little improvement.

The filtrate factor preparation which was used was assayed[2] for B_6 and flavin by being fed at two levels with flavin to one group of rats and with B_6 to another. No improvement was found in the latter by doubling or quadrupling the quantity of filtrate factor, which was taken to mean that the latter contained little if any flavin. Definite improvement in growth, sufficient to make these groups equal in performance to those receiving the standard dose of B_6 supplement, resulted however when the filtrate factor was doubled and quadrupled for the group given flavin but no B_6. Apparently the filtrate factor in the quantity fed, 0.5 cc. per day, contained one-half the amount of B_6 in the daily dose of wheat germ preparation used as source of B_6, that is one-half of the equivalent of 0.5 gm. wheat germ. It is not surprising therefore that when filtrate factor and flavin were given, fairly good growth resulted on all diets.

We were puzzled by the good growth resulting from the addition of flavin and B_6 to the cornstarch diet and by the relative ineffectiveness of additions of filtrate factor either alone or with the other supplements, as shown by the growth curves in figure 3. The supposition must be made either that the cornstarch carries appreciable amounts of filtrate factor or that it promotes intestinal formation of this factor. Since the B_6 preparation appeared to be free from filtrate factor by chick assay and by its effect with the sucrose and lactose diets, no other source of the filtrate factor than the starch can be found.

Hogan and Richardson ('34) have reported cure of florid dermatitis in rats fed a sucrose diet by concentrated hot

[2] These assays were made by Dorothy V. Rundle.

alcohol extracts of cornstarch, the condition cured being possibly due at least in part to filtrate factor deficiency. Certainly the factor which might be expected to adhere to cornstarch is not the filtrate factor which has begun to be identified through clinical cures of pellagra and blacktongue with the P-P of Goldberger. Several recent reports moreover, emphasize the relative poverty of cereals in general and maize in particular in all these factors except B_6.

Many of the studies with rats in which pellagra-like conditions were produced have been carried on with basal diets containing large amounts of cornstarch, rice starch or cereal mixtures. The work of Birch, György and Harris ('35) which led to their conclusion that rats do not need or can synthesize the third factor, that is additional to flavin and B_6, was done with a diet containing 68% cornstarch. If the filtrate factor is not actually carried by the starch usually employed, it may be that the bacterial flora favored by the presence of the starch in the rat's intestine synthesize it.

The obvious inconsistency of the presence of the filtrate factor in cornstarch along with the role of corn (maize) as a prominent part of pellagra-producing diets raises again the question of the specificity of the filtrate factor as the pellagra-preventing substance or at least of the singleness of character of this accessory or accessories.

THE EFFECT OF SUCROSE

The rats fed the sucrose diets supplemented with B_1 only developed dermatitis regularly if they survived long enough, grew not at all and usually died in a few weeks. Some growth resulted when any one of the three factors was given, the filtrate factor probably because of its B_6 contamination being most effective (fig. 4). When any two of the factors were given together, improvement over that seen on any one alone resulted, and when all three were given, normal animals were produced nearly as large as those given 0.5 gm. yeast daily. The improved growth obtained by the addition of each factor is in almost mathematical relation to the growths obtained

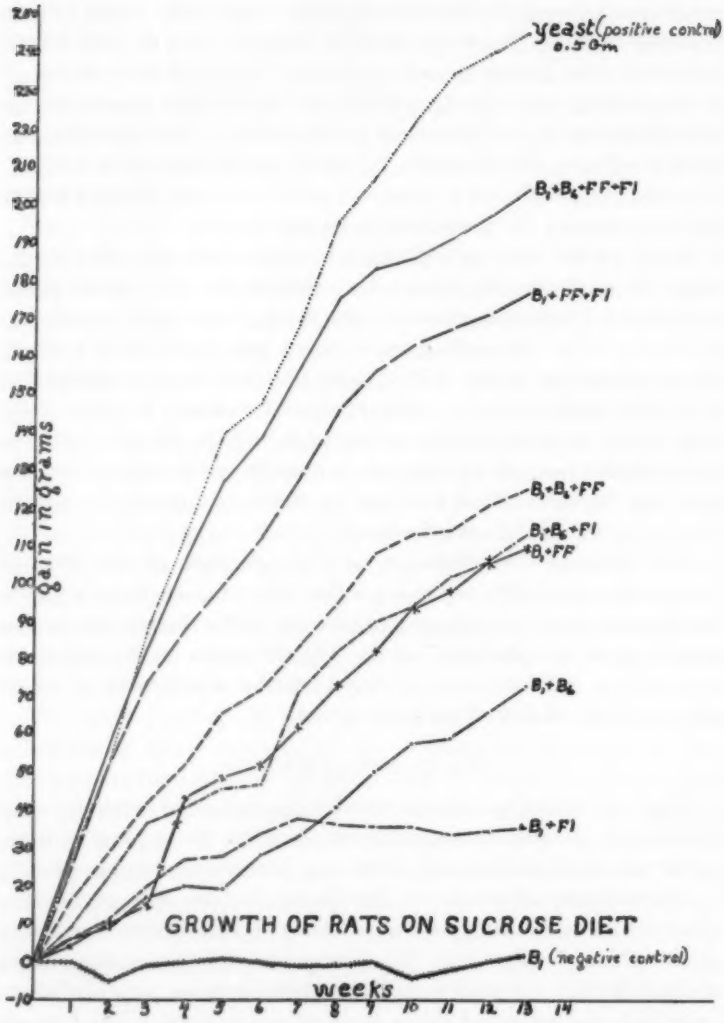

Fig. 4 Growth of young rats on sucrose diet with one, two, three or all four of the B vitamins: B_1, flavin (Fl), filtrate factors (FF), vitamin B_6 (B_6).

when one or two are progressively missing. Thus in 14 weeks flavin alone promoted an average gain of 35 gm., B_6 alone, a gain of 72 gm., filtrate factor alone, a gain of 108 gm. In the latter case it must be remembered that about one-half the standard dose of B_6 was present in the filtrate factor supplement. When flavin and B_6 were given together, the gain was 112 gm., almost an exact addition of the separate gains. When flavin and filtrate factor were given together, the gain was 177 gm., close to the normal obtained with flavin, B_6 and filtrate factor, 204 gm. When B_6 and filtrate factor were given together, the gain was 125 gm., 73 of this ascribable to the B_6. Apparently the sucrose diet is completely free from all of these factors and also produces none of them in the intestine. It would seem therefore to be the carbohydrate of choice for basal diets for rats in the study of the vitamin B_2 complex. This conclusion was reached also by Halliday and Evans ('37) who found the depletion period shorter and deficiency symptoms more severe with sucrose instead of cornstarch diets.

The studies of Guerrant, Dutcher and Tomey ('35) with diets containing various carbohydrates and deficient in all the B vitamins have indicated the synthetic activities of the flora promoted by dextrin and to a slighter extent, lactose. The overwhelming effect of vitamin B_1 deficiency apparently obscured the effect upon the B_2 factors in this case. The recent work of Bender, Ansbacher, Flanigan and Supplee ('36) has differentiated the effect of dextrin however since B_1 and lactoflavin were supplied. No dermatitis occurred on the dextrin ration but with a similar sucrose diet dermatitis was quite severe. The addition of rice polish concentrate cured the dermatitis in the latter animals and produced practically normal growth. But a flavin-free milk concentrate had no such effect. Whether the addition of the milk concentrate improved the growth of the dextrin-fed rats is not evident and there is no statement as to the use of the rice polish concentrate with the dextrin ration. It is impossible to judge therefore whether the dextrin provides both B_6 and filtrate factor in adequate amounts. Obviously it does not provide

flavin as does the lactose. There is apparently some difference between the intestinal activities or the original contaminations of these two carbohydrates. It seems clear however from the absence of dermatitis in both lactose and dextrin-fed rats that B_6 is produced in both cases.

BLOOD AND URINE ANALYSES

As determined by the method of Shaffer and Somogyi ('33) the blood sugar of the rats on the sucrose diet was within the usual normal range, 97 to 121 mg. per 100 cc., in all cases both with and without deficiencies. The same was true of those on the cornstarch diet in which the range was 108 to 125 mg. per 100 cc. Those on the lactose diet however had blood reducing sugar values of 158 to 241 mg. per 100 cc. Such high blood sugar values are in agreement with the findings of Day ('36) and of Mitchell, Merriam and Cook ('37). No attempt was made to differentiate glucose from galactose in the blood of the lactose-fed rats. A few animals on lactose plus vitamin B_1 were given small frequent doses of insulin zinc for 3 or 4 weeks in order to determine whether the cataracts produced by such a diet might be thus averted. No change in severity or early appearance of the cataracts resulted but the reducing sugar in the blood of these animals was found to be lowered to the same extent as in cornstarch-fed animals. This observation is contrary to the report of Mitchell, Merriam and Cook ('37), who found blood galactose, assuming that most of the hyperglycemia was due to galactose, unaffected by insulin.

Urine sugars as determined in samples of undiluted rat urine collected with special precautions to avoid contamination were found to be lowest on the starch diet, 44 to 117 mg. per 100 cc. of urine, intermediate on the sucrose diet, 159 to 480 mg. per 100 cc. of urine and highest on the lactose diet, 254 to 956 mg. per 100 cc. of urine. With sucrose the larger urine sugar output was seen on the deficient diets.

Urine calcium was determined also on the undiluted urine and was found to be largest on lactose diet, 59 to 171 mg.

per 100 cc. of urine. On the starch and sucrose diets the range of excretion was the same, 3 to 27 mg. per 100 cc. of urine. This effect of the lactose is perhaps only the result of the increased calcium absorption first reported by Bergeim ('26) as resulting from the presence of lactose in the intestine.

THE GRAYING OF RAT HAIR

An interesting symptom which developed in many of the filtrate factor-deficient animals was the graying of hair in regular patterns. This was most evident in the black rats but could be seen in gray and hooded animals as well. In very few cases was the graying evident in those which received only vitamin B_1. The rats which grew well and were relatively free from dermatitis or alopecia in nearly all cases became gray. These were chiefly the rats on sucrose diet which received no filtrate factor. Those on cornstarch in many cases grayed also but only after a prolonged period. The phenomenon was first observed in animals given the starch diet and small amounts of wheat germ, and all of these were cured of the depigmentation by the filtrate factor preparation from rice bran. In all cases when B_6 was present in the diet as well as flavin the graying was most severe.

Three black rats of the same litter fed the sucrose diet are shown in figure 5. Rat no. 10 was given all three factors throughout the experiment which continued for 24 weeks. This animal was normal in all respects at all times and continued to have a glossy black coat. Rat no. 11 was given the B_6 preparation only for the first 12 weeks during which time its fur became streaked with gray. The filtrate factor preparation was then added in standard amount to the diet of no. 11. After 8 weeks the fur of this animal had again become glossy and black. Rat no. 12 was given B_6 and flavin only for these 20 weeks with increasing graying of the fur, bloody ears and nose and emaciation. During the last 8 weeks 0.17 mg. of copper as copper sulfate was given rat no. 12 daily. The photograph shown in figure 5 was taken after the experiment had progressed for 20 weeks. Obviously the two rats

given the filtrate factor have normal fur while the rat lacking the filtrate factor is in poor condition with completely depigmented fur. The copper had no curative effect on the depigmentation. Gorter ('35) has recently claimed that the depigmentation is due to lack of copper and that it may be prevented or cured by the administration of minute amounts of copper

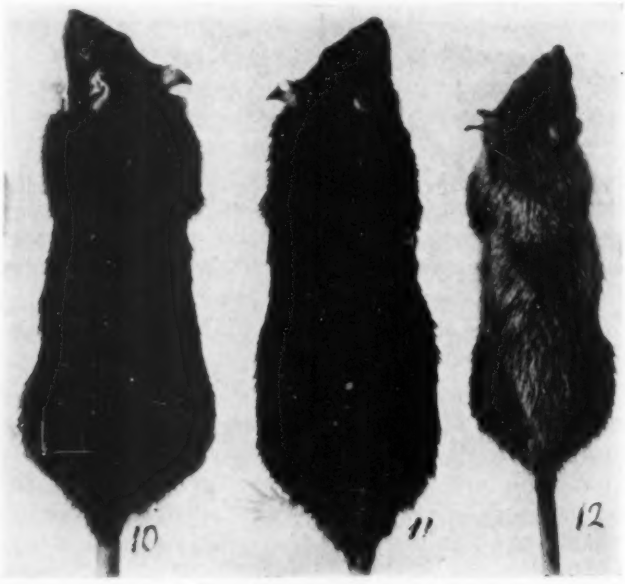

Fig. 5 Littermate black rats on the sucrose diet. No. 10 was given all of the B_2 vitamins throughout the experiment which continued for 24 weeks. No. 11 was given B_6 only for 12 weeks, then filtrate factor. No. 12 was given B_6 and flavin (no filtrate factor) but was given additional Cu latter part of experiment.

salts. The specificity of copper may be questioned in these cases since all rats in this laboratory obtain their water from bottles equipped with pure copper drinking tips. After the photograph shown in figure 5 was taken, rat no. 12 was given the filtrate factor in double the standard daily dose for 4 weeks at which time its fur had become black again, it had increased in size and had become normal in all respects.

Another group of some forty young black rats have been placed on the sucrose diet with vitamins B_1 and B_6 and flavin but without filtrate factor. Nearly all of these animals have shown marked graying of the fur within 8 to 12 weeks and rapid resumption of the normal color when filtrate factor preparations made from rice bran and from liver were fed. Control animals given the filtrate factor from the beginning have retained normal fur color. We conclude that the filtrate factor or factors prevents and cures graying of rat hair.

SUMMARY

When lactose in sufficient amount is incorporated in the basal diet, of the three necessary factors now recognized in the vitamin B_2 complex, both flavin and vitamin B_6 need not be given separately and are apparently elaborated in the intestine of the animals. The filtrate factor however must be given in order to obtain normal growth. In no case did dermatitis develop in lactose-fed rats even when the proportion of lactose was lowered to 30%, but a large proportion of the rats developed cataracts. The severity of the cataract occurrence was mitigated when all the vitamin B factors were supplied, and this prophylactic effect seemed to inhere in the filtrate factor particularly.

When cornstarch is used as the carbohydrate of the basal diet, dermatitis and relative failure of growth result unless B_6 and flavin are supplied. The filtrate factor appears to be present in limited amount in the starch or to be produced in the intestine when cornstarch is used. No cataracts occurred on any of the starch or sucrose diets.

When sucrose is used as the carbohydrate of the basal diet, earlier and more severe dermatitis and growth failure occur unless all three of the factors are supplied. Better growth response was obtained with sucrose than with starch when one or more of the factors was supplied and better response with starch than with lactose.

Graying of the hair of black rats was seen on most of the diets which lacked the filtrate factor and this was prevented or

cured by administration of the rice bran concentrate which was used as source of the filtrate factor.

Blood and urine reducing sugar of the rats on lactose were much increased over that of the starch-fed group. Urine calcium was also much increased in this group. The sucrose diets produced normal blood sugar values but when one or more of the B_2 factors was lacking, high urine sugars. Some clue to the mode of action of these factors may be found in this behavior.

CONCLUSIONS

Lactose favors the production in the intestine of rats, probably by microorganisms, of both flavin and vitamin B_6 but not the filtrate factor.

Cornstarch either carries with it or favors the production of the filtrate factor only.

Sucrose neither carries nor produces any of the vitamin B_2 factors and is therefore the carbohydrate of choice for the study of these factors.

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ETIOLOGY OF SEBACEOUS GLAND ATROPHY IN THE RAT IN AVITAMINOSIS [1]

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EIGHT FIGURES

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INTRODUCTION

Albino rats on vitamin G [2] deficient diets regularly show atrophy of the sebaceous glands and thinning of the epithelium as demonstrated by microscopic sections of the tail (Smith, '32). These findings have been confirmed and extended by Smith and Sprunt ('35) who showed that the lesions were independent of the initial age of the rat or the season of the year. The changes in the sebaceous glands and epithelium of the tail were demonstrated in the living animals by biopsy. Autoclaved yeast was uniformly effective both in preventing and in curing this symptom complex.

The same type of epithelial and sebaceous gland lesion occurred also in rats on vitamin A and vitamin B deficient diets, but was less severe. The lesions did not occur on Steenbock and Black's rickets-producing diet no. 2965. It has been suggested by Smith and Sprunt ('35) that the changes noted in rats on vitamin A and B deficient diets might be due to a secondary shortage of vitamin G precipitated by insufficient intake of food or faulty absorption of this factor from a basic diet. The present study was designed to test this assumption.

[1] Part of the expenses of this work was covered by grants from the Rockefeller Foundation, New York, N. Y., and the Lederle Laboratories, Pearl River, N. Y.

[2] Vitamin G as used in this report refers to all the factors of the complex occurring in autoclaved yeast.

EXPERIMENTAL

Animals. Young albino rats of the Wistar strain were used. The ages ranged from 27 to 33 days. They were housed in experimental cages with raised screen bottoms.

Grouping. Two groups of rats were used. Group I, consisting of forty-nine rats, served for the vitamin A study and group II, consisting of 135 rats, served for the vitamin G study. Each group was subdivided into three groups a, b and c, for the purpose of contrasting the two deficiencies under slight dietary variations.

1. Group I-a (seventeen rats) was placed on a basal diet deficient in vitamin A, to which vitamin G was added. Group II-a (thirty-five rats) was placed on a basal diet deficient in vitamin G to which vitamin A was added. This was the type of diet used in our previous studies. Under the influence of the original deficiency the animal's food intake decreases, so that one or more secondary deficiencies might be expected. To test this possibility, the next variation was made.

2. Groups I-b (twenty-one rats) and II-b (fifty-five rats) were placed on vitamin A and vitamin G deficient diets exactly as were the rats in groups I-a and II-a. In this experiment, however, the vitamin G of the A-deficient diet and the vitamin A of the G-deficient diet were fed individually, not mixed with the basal diet. The level of intake of these supplementary vitamins was thus maintained even after food consumption began to decrease.

3. Groups I-c (eleven rats) and II-c (forty-five rats) were positive control groups receiving diets identical with those of groups I-a and II-a except that group I-c received in addition a source of vitamin A and group II-c a source of vitamin G.

Diets. The A-free diet, A-a,[3] fed to group I-a, consisted of casein (hot alcohol extracted) 18%, Osborn and Mendel salt mixture 4%, dry brewers yeast 10%, cornstarch 67%, sodium chloride 1%, and 6 drops of viosterol (250-D) per kilo of diet.

[3] The large letter indicates the vitamin deficiency and the small one the group receiving it.

The diet A-b, fed to group I-b, had approximately the same ingredients except that crystalline vitamin B [4] and autoclaved yeast replaced the untreated yeast. This was done to maintain the level of vitamin G as autoclaved yeast. The minerals 4%, casein 20% and starch 76% were mixed in the basal diet and the yeast and vitamin B were fed separately.

TABLE 1

Diets

INGREDIENTS	A-a	A-b	A-c	G-a	G-b	G-c
	%	%	%	%	%	%
Casein hot. alc. extd.	18	18	18			
Casein alc. extd.				18	18	18
Cornstarch	67	78	67			
Osborn and Mendel salts	4	4	4	4	4	4
Brewers yeast	10		10			
Irradiated ergosterol (250-D)	0.5 gm. per kilo of diet					
Sodium chloride	1		1			
Sucrose				68	70	68
Butter fat				8	8	8
Cod liver oil				2		2
Daily individual feeding						
Crystalline vitamin B		20 γ		20 γ	20 γ	20 γ
Autoclaved yeast		0.5 gm.				0.5 gm.
Cod liver oil			2 drops		2 drops	
Irradiated ergosterol		1 drop (w)[1]				
Lactoflavin				20 γ	20 γ	20 γ

[1] w = weekly.

Diet A-c, fed to group I-c, was identical with diet A-a except that each rat in this group received in addition 2 drops of cod liver oil daily.

The G-deficient diet G-a, fed to group II-a, consisted of casein (alcohol extracted) 18%, Osborn and Mendel salt mixture 4%, butter fat 8%, cod liver oil 2%, sucrose 68%, mixed into the basal diet and supplemented with pure crystalline vitamin B and purified lactoflavin [5] fed individually to each rat.

[4] Furnished by the Merck Chemical Co.

[5] Furnished by the Winthrop Chemical Co.

The G-deficient diet G-b, fed to group II-b, was identical with diet G-a except that the cod liver oil was fed individually to each rat in order to maintain the level of vitamin A.

Diet G-c, fed to group II-c, was identical with diet G-a except that autoclaved yeast was fed in addition (table 1).

Method of giving supplementary feedings

Cod liver oil. Two drops daily of a good grade of oil was fed through a medicine dropper.

Autoclaved yeast. Brewers yeast was autoclaved 2½ hours at 15 pounds pressure and 0.5 gm. of the dry product was placed in a small dish and fed daily.

Vitamin B. Ten milligrams of the natural crystals were dissolved in 25 cc. of distilled water and 1 drop of this solution, equivalent to 20 γ of the solid product, was fed daily to each rat.

Lactoflavin. One drop, equivalent to 20 γ pure lactoflavin, was fed daily to each animal.

Vitamin D. One drop per rat per week of viosterol (250-D) was given.

At the end of the experiment, or at death of any individual, cross sections of the tail were made at the base, mid-portion and tip. The details of the method have been published in a previous communication (Smith and Sprunt, '35).

Fig. 1 Photomicrograph of cross section of tail of rat in group I-a. Note thinning of epithelium and atrophy of the sebaceous glands. × 150.

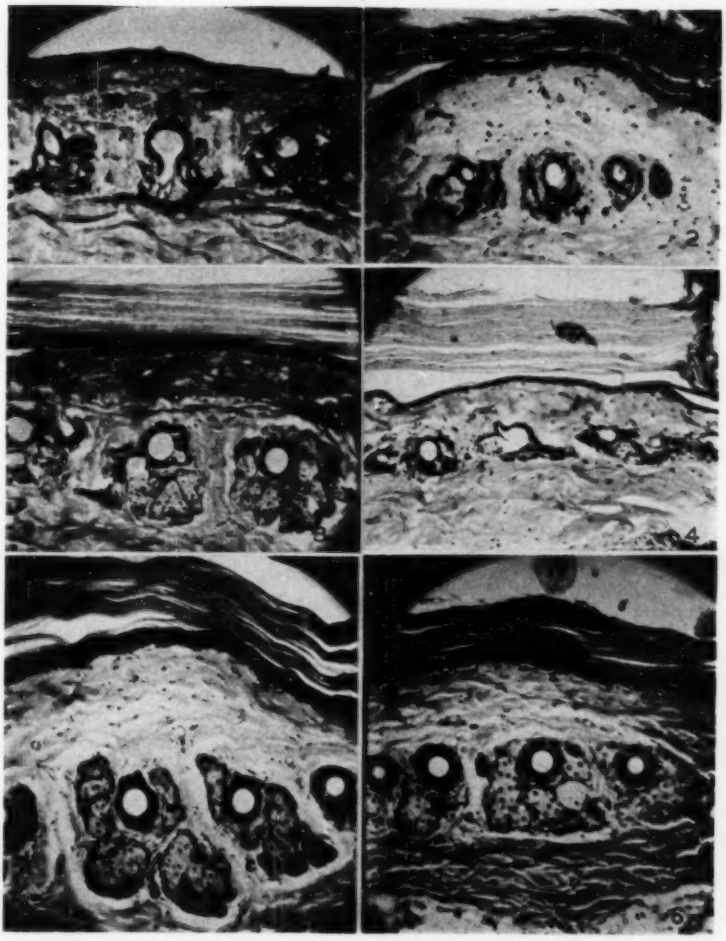
Fig. 2 Photomicrograph of cross section of tail of rat in group II-a. Note thin epithelium and almost completely atrophied sebaceous glands. × 150.

Fig. 3 Photomicrograph of cross section of tail of rat in group I-b. These sebaceous glands are almost normal. × 150.

Fig. 4 Photomicrograph of cross section of tail of rat in group II-b. There is marked thinning of the epithelium and almost complete atrophy of sebaceous glands. × 150.

Fig. 5 Photomicrograph of cross section of tail of rat in group I-c. Positive control. Epithelium and sebaceous glands are normal. × 150.

Fig. 6 Photomicrograph of cross section of tail of rat in group II-c. Positive control. Both epithelium and sebaceous glands are normal. × 150.



Figures 1 to 6, microscopic sections of rat tails. 1, rat on A free diet with G mixed into basal; 2, rat on G free diet with A mixed into basal; 3, rat on A free diet with G fed separately; 4, rat on G free diet with A fed separately; 5, rat on A free diet + A (positive control); 6, rat on G free diet + G (positive control).

RESULTS

Group I. Vitamin A study

1. The rats on diet A-a grew at a practically normal rate for 9 weeks, maintained a plateau for 2 weeks and then the weight declined rapidly until death (fig. 7). Coincident with

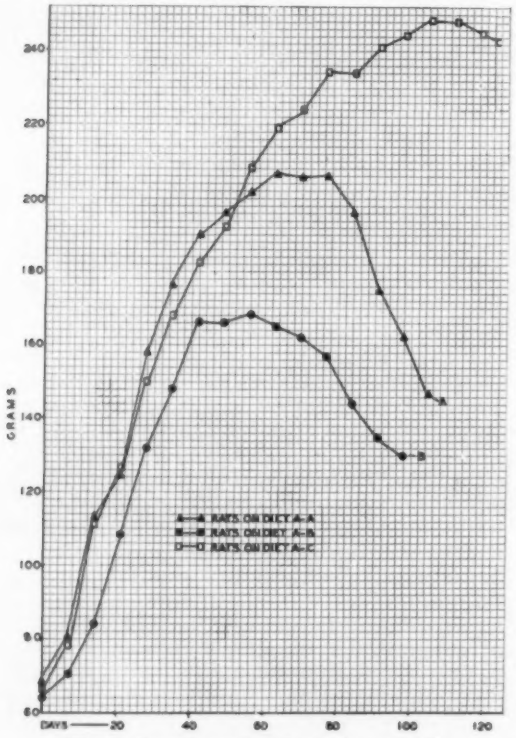


Fig. 7 Growth curves of rats in group I.

the decline in weight, symptoms of A deficiency developed, namely, somewhat roughened coat, xerophthalmia, and an unsteady gait. At autopsy fourteen out of seventeen rats had a large purulent abscess at the base of the tongue. The microscopic sections of the tail made at this time showed some atrophy and slight thinning of the epithelium (fig. 1).

2. The rats on diet A-b grew at a rate slightly less than normal for the first 6 weeks, showed little or no gain for 2 weeks followed by a gradual loss of weight until death. The development of eye symptoms, rough coat and unsteady gait, characteristic of vitamin A deficiency, coincided with the failure of growth. At autopsy thirteen of twenty-one rats had a large purulent abscess at the base of the tongue. The

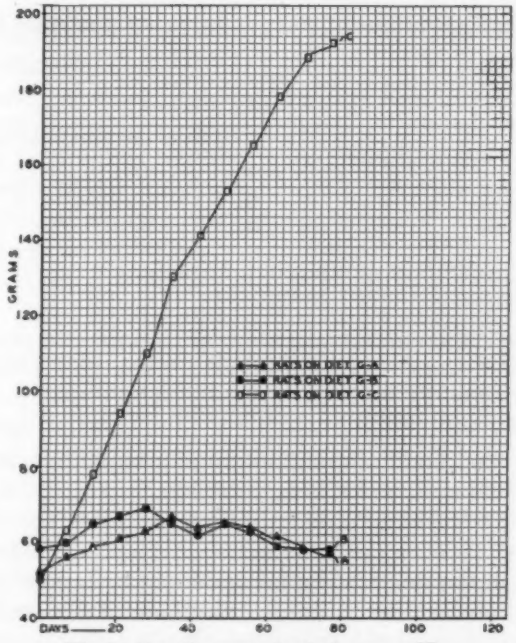


Fig. 8 Growth curve of rats in group II.

microscopic sections of the tail showed almost normal sebaceous glands and epithelium (fig. 3), evidence, we think, of the protective effect of vitamin G, as supplied by the autoclaved yeast.

It will be noted that the rats on diet A-a grew consistently better than the rats on diet A-b. This can be accounted for probably on the assumption that the autoclaved yeast and

crystalline vitamin B as fed to the animals do not provide exactly the same vitamin B and G factors that are found in untreated yeast. However, the factor missing or deficient is not significant in the present study, since the rats in group b, though growing at a slower rate, had the more normal sebaceous glands.

3. The rats on diet A-c grew normally (fig. 7) and remained symptom-free throughout the experiment.

Microscopic sections of the tail revealed a normal histology (fig. 5).

TABLE 2

Sebaceous gland lesions

GROUP	DIET	TOTAL NUMBER OF RATS	EXTENT OF SEBACEOUS GLAND LESION			
			Normal	+[1]	++[2]	+++[3]
I-a	A-a	17	0	7	10	0
I-b	A-b	21	14	5	1	1
I-c	A-c	11	11	0	0	0
II-a	G-a	35	0	1	14	20
II-b	G-b	55	0	3	11	41
II-c	G-c	45	43	0	2	0

[1] Slight lesion. [2] Moderate lesion. [3] Severe lesion.

Group II. Vitamin G study

1. The rats on diet G-a grew very poorly from the beginning and after 5 weeks lost weight gradually until the end of the experiment (fig. 8). Dermatitis, affecting the nose, mouth and all four paws, occurred regularly. Priapism was observed in the males and a failure to open the vaginal orifice in fifteen of eighteen females. Sections of the tail showed marked atrophy of the sebaceous glands and thinning of the epithelium (fig. 2).

2. The rats on diet G-b also grew very poorly (fig. 8) and exhibited the same symptoms as the rats on diet G-a. Sections of the tail showed marked atrophy of the sebaceous glands and thinning of the epithelium (fig. 4), thus indicating that no protection was afforded by feeding individually vitamin A as cod liver oil.

3. Rats consuming diet G-c grew normally (fig. 8) and appeared healthy. They exhibited no gross symptoms. Most of the females were pregnant at the end of the experiment. The tail sections revealed well-developed sebaceous glands and normal epithelium (fig. 6). (See table 2 for comparative histological findings.)

Vitamin B study

We could not contrast directly the effect of vitamin B with that of vitamin G, as we did in the case of vitamin A, owing to the early onset of anorexia, which interferred with the consumption of the autoclaved yeast. However, in a group of sixty rats fed crystalline vitamin B individually, while consuming a vitamin G deficient diet, there was no evidence of protection against the development of epithelial and sebaceous gland lesions.

DISCUSSION

Wolbach and Howe ('25 a and b) found slight atrophy of the sebaceous glands in rats on an A-deficient diet. As in group I-a, their dietary ingredients were all mixed into the basal diet. Our results with this type of diet correspond very closely to theirs. In our studies, however, when vitamin G in the form of autoclaved yeast was fed separately in group I-b, there was marked protection of the sebaceous glands. On the other hand, the feeding of vitamin A separately to rats in group II-b, on a G-deficient diet, failed to protect the sebaceous glands.

Much has been written about alterations in the sebaceous glands of human skin. These changes have been variously termed seborrhoeic dermatitis, phrynoderma, toad skin, permanent goose skin, etc. Such lesions have been observed in association with pellagra (Bigland, '20; Biggam and Ghalioungui, '33; Corkill, '34; Stannus, '12; Castellani and Chalmers, '19; Smith and Sprunt, '35). Castellani and Chalmers describe seborrhoeic changes in pellagra as a "frequency of nasal or facial seborrhoea, which is to be especially

noted in the nose where the sebaceous follicles are very prominent and filled with plugs of sebaceous material."

Similar lesions occur also in association with keratomalacia (Pillat, '29; Frazier and Hu, '31; Nicholls, '31; Loewenthal, '33; Mackay, '34; Goodwin, '34; Aykroyd and Krishmann, '37; Eddy and Dalldorf, '37; Rao, '37). Rao in his excellent report gives a very good clinical and histo-pathological description of these skin changes which he attributed to a nutritional deficiency in which vitamin A is an important factor. The sites affected were mainly "the extensor surfaces of the arms, thighs and upper part (postero-lateral aspect) of the forearms near the elbow. Histopathologically the condition was characterized by a superficial non-inflammatory hyperkeratosis of the epithelium of the epidermis and hair follicles, distension of the mouths of the pilo-sebaceous follicles by horny plugs, atrophy of the sebaceous glands and impaired function of the sweat glands." Rao stated that the atrophy of the sebaceous glands appeared to be secondary to the hyperkeratinization of the epidermis and hair follicles.

In the present study atrophy of the sebaceous glands of rats resulted from a deficiency of vitamin G either as a primary deficiency or as a secondary deficiency conditioned by an inadequate supply of vitamin A.

SUMMARY

A study has been made of the part played by a deficiency of vitamins A and G as well as vitamin B in producing skin changes involving thinning of the epithelium and atrophy of the sebaceous glands in rats.

1. The skin alterations described above, and demonstrated by microscopic section of the tail, occur regularly in rats on a diet deficient in vitamin G.

2. Similar but less marked changes occur on vitamin A and vitamin B deficient diets if the source of G is mixed into the diet.

3. The sebaceous glands are almost normal, being only slightly altered, on a vitamin A deficient diet if the level of vitamin G is maintained by feeding the autoclaved yeast separately.

4. The sebaceous glands are not protected, however, on a vitamin G-deficient diet when the level of vitamin A is maintained by feeding cod liver oil separately.

5. The sebaceous glands are not protected on a vitamin G deficient diet when the level of vitamin B is maintained by feeding the crystalline material separately.

CONCLUSION

Atrophy of the sebaceous glands in the rat's tail is apparently due to a deficiency of some factor of the vitamin G complex.

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RIBOFLAVIN AND A FURTHER GROWTH ESSENTIAL IN THE TISSUES

QUANTITATIVE DISTRIBUTION AND THE INFLUENCE OF THE FOOD

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TWO FIGURES

(Received for publication June 25, 1937)

Without asking space to review the literature of the distribution in the tissues of the nutritionally-undifferentiated group of substances which give flavin reactions in vitro, we would here record briefly the results of feeding experiments made to ascertain 1) the quantitative distribution of riboflavin (lactoflavin, 'vitamin G') in the tissues of favorably nourished animals, and 2) the influence of the level of intake of riboflavin and a further growth essential upon the concentrations of these substances in the tissues. In the interest of brevity and clarity, we first present the experimental findings, and then discuss such previous work as bears upon the phenomena with which we are here concerned.

EXPERIMENTAL

The general plan of these experiments was similar to that followed in the previous work of this laboratory upon the distribution of vitamin A in the tissues (Sherman and Boynton, '25). Healthy young adult rats of known nutritional history were slaughtered, dissected at once, and weighed amounts of different tissues fed as sole source of riboflavin to test animals. For the quantitative measurement of the relative amounts of riboflavin thus found, the method described by Bourquin and Sherman ('31) was used, other

work in this laboratory having shown that riboflavin (lactoflavin) is the growth limiting factor under the conditions of this method (Booher, '33, '34; Bisbey and Sherman, '35) and that the 'vitamin G values' as measured in this laboratory are measures of riboflavin.

The animals whose tissues were under investigation were chloroformed and bled from the heart to obtain samples of blood for feeding and to free the tissues from surplus blood. Skeletal muscle was quickly removed, organs were dissected out with the minimum of connecting blood vessels and any adhering drops of blood were removed by absorption with filter paper; then weighed portions of the organs and tissues were fed immediately to preclude deterioration of riboflavin value. The material thus fed was always readily and completely consumed by the test animals. Tissue material was thus obtained and fed thrice weekly; but for convenience of comparison and interpretation the data are given on the usual basis of 'daily except Sunday' allowance, i.e., 'per day' meaning one-sixth of the actual amount consumed in the course of each week.

Figure 1 shows the average weight curves resulting from the feeding of the skeletal muscle, heart muscle, kidney and liver of rats which had been reared on our diet B or diet 13, a mixture of two-thirds ground whole wheat and one-third dried whole milk with table salt and distilled water. This food mixture contains about 6 γ of riboflavin per gram. Each curve represents the average record of from three to fourteen test animals. Blood, brain and spleen were fed to smaller numbers. The findings are therefore more quantitative for the four first-named, than for the three last-named, tissues. Curves 1 to 4 of figure 1 indicate about equal amounts of riboflavin in 1.5 gm. skeletal muscle, 0.3 gm. heart muscle, or 0.15 gm. of kidney or of liver. But in a comparison at a lower level of feeding (curves 5 and 6 of fig. 1) it appears that distinctly more riboflavin was contained in 0.075 gm. liver than in 1.0 gm. skeletal muscle. Thus these data indicate that the concentration of riboflavin was about five times

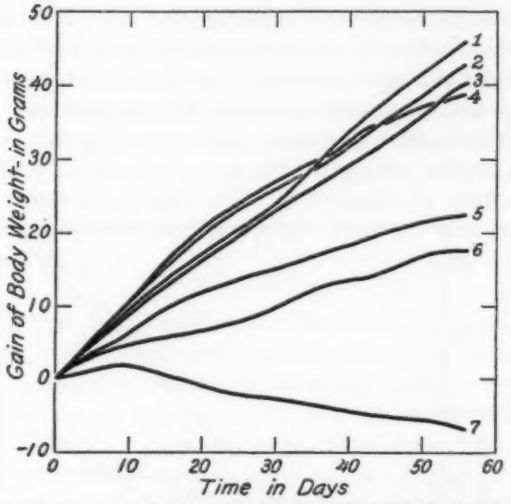

Fig. 1 Growth curves measuring distribution of riboflavin in the tissues of diet B rats: 1) From the feeding of 0.3 gm. per day of heart muscle; 2) of 0.15 gm. liver; 3) of 0.15 gm. kidney; 4) of 1.5 gm. muscle; 5) of 0.075 gm. liver; 6) of 1.0 gm. muscle; 7) negative controls.

as high in heart muscle, about ten times as high in kidney, and ten to twenty times as high in liver, as in skeletal muscle. Weight for weight, brain and spleen appeared to contain about two to three times as much, and blood only one-third as much, as skeletal muscle.

The basal diet which was fed the test animals in these measurements of the riboflavin values of tissues was the diet 555 of the accompanying table 1.

TABLE 1

Composition of diets 429 and 555

	Diet 429	*Diet 555*
Casein (extracted with 60% alcohol)	9	18
Osborne-Mendel salt mixture	2	4
Butterfat	9	8
Cod liver oil	1	2
Skim milk powder	30	..
Whole wheat (ground)	40	..
Cornstarch	9	68[1]
Alcohol extract of ground wheat	..	See text

[1] Including the wheat extract.

In a second series of experiments the same method of testing was applied to muscle and liver of rats which had been reared from the age of 28 days to that of 64 to 66 days on diet 555, while parallel animals for comparison were fed through this same age period with diet 429, the composition of which is also shown in table 1.

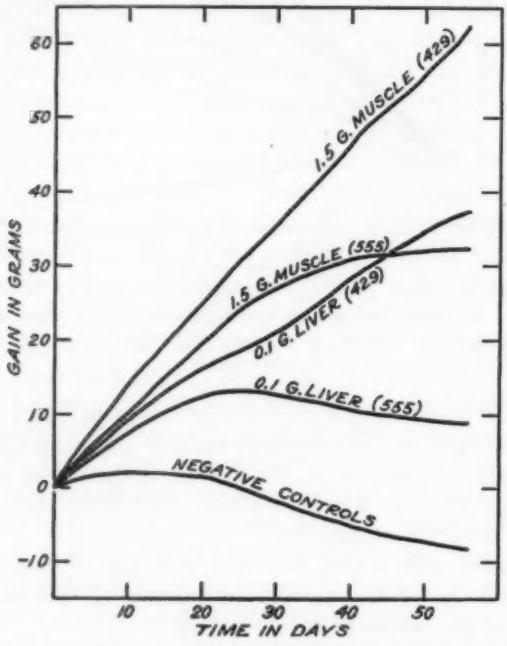

Fig. 2 Growth curves of experiments comparing the riboflavin contents of the tissues of animals from riboflavin rich diet 429 and riboflavin poor diet 555.

In the case of diet 555 the cornstarch carried an 80% alcohol extract of 60 gm. ground whole wheat to every 100 gm. of the air dry diet. This contains practically no riboflavin, and provides enough of all other water soluble vitamins needed by the rat for the amount of growth involved in our ordinary measurements of riboflavin values.

From figure 2 it will be seen that the tissues of rats reared on diet 429 (excellently adapted to all the nutritive requirements of rats) supported good growth consistently throughout the 8-week test period; while the results of feeding equal amounts of the corresponding tissues of rats reared on diet 555 (deficient in riboflavin) showed that the tissues of these latter rats contained distinctly less riboflavin than those of the rats whose food had furnished more of this substance.

DISCUSSION

The data just mentioned show also that the tissues of the animals from riboflavin deficient diet resulted in less consistently sustained growth. This latter finding may be explained in either of two ways. 1) As all of the tissues fed were highly relished by the test animals it may be that the feeding of even the riboflavin poor tissues stimulated a temporary increase in weight of the test animals somewhat greater than that properly attributable to their riboflavin intake alone. 2) It is equally conceivable that the early part of the weight curve is the valid measure of the riboflavin content of the tissues fed, and that the later flattening of the curve is due to the developing shortage of a second growth limiting factor of which the tissues of the diet 429 rats furnished more than did those of the rats from diet 555, while the tissues of the test animals contained enough for the support of their growth for a short time only, after which the curve flattens because of shortage of this further factor. This would be in line with the experience of Coward, Key and Morgan ('29) and of Chick and Copping ('30); and so our 'further factor' may be identical with the Coward factor, or Chick and Copping's 'Y factor' or both. It may also be identical with vitamin B_6. Of this, Copping ('36) finds only the lesser part extracted by 80% alcohol from ground cereals, so that in our diet 555 there may have been sufficient vitamin B_6 for only limited growth.

As present knowledge does not permit a confident choice between the foregoing alternatives, we have not a precise

quantitative measure of the riboflavin content in the tissues of the diet 555 rats; but it is clearly established that the level of riboflavin intake does exert a considerable influence upon the riboflavin concentration in the muscles as well as in the liver.

While riboflavin, like vitamins A, B_1 and B_6, occurs in greater concentration in the liver than in the muscles, the quantitative relationship varies widely. Sherman and Boynton ('25) found 200 to 400 times as much vitamin A per gram of liver as of muscle, while for the same species on the same diet Brodie and MacLeod ('35) found only a tenfold higher concentration of vitamin B_1, and we here find only from ten to twenty times as high a concentration of riboflavin in the liver as in the muscles. Working with a different species (beef) György ('35) likewise found for riboflavin a concentration in liver about fifteenfold higher than in muscle, while the concentration of vitamin B_6 was about one-third as high in muscle as in liver. The very low vitamin A content of muscle has not been found to be measurably influenced by the level of intake of this vitamin in the food, while this does have a very great influence upon the amount found in the liver, apparently because any surplus absorbed from the food is chiefly laid by in the liver, presumably in relatively passive storage. In the present experiments the level of intake of riboflavin has influenced the concentrations in the liver less and in the muscles more than was the case with vitamin A. This is consistent with the view that relatively less of riboflavin than of vitamin A is laid away in passive storage in the liver, and that relatively more of whatever surplus the food furnishes is distributed to the tissues generally, and raises their riboflavin content. In the experiments of Brodie and MacLeod ('35) increased intake of vitamin B_1 seemed to increase the concentration of this vitamin in muscle, liver, kidney and brain in about the same relative proportion. Thus there is some measure of similarity in quantitative distribution between the two water soluble vitamins, B_1 and riboflavin, as contrasted with the fat

soluble vitamin A. It also appears that the tissues tend, under stress of shortage to conserve their riboflavin more retentively than their vitamin B_1.

The findings for riboflavin as compared both with those for vitamin A and for vitamin B_1, tend therefore to confirm the general concept of riboflavin as an imperatively essential constituent of the tissues generally; and which when liberally supplied by the food reaches distinctly higher concentrations in the tissues than when the intake is of only minimal adequacy.

The present experiments might also be interpreted to support the view that muscle, liver, and milk (and Osborne and Mendel's 'natural protein-free milk') have special growth promoting value either because of factors not yet fully known or because of supplementary relations which exist among known factors but have not yet been fully explored. This is consistent with the differences between the growth curves shown in figure 2 as supported by feedings of corresponding tissues from rats differently fed, whichever of the alternative interpretations above suggested is considered the more probable.

We have no desire to increase the number of postulated nutritional essentials in the vitamin B complex. Rather it is to be hoped that these observations may ultimately be so integrated with those made in connection with the Coward factor (Coward, Key and Morgan, '29), factor Y (Chick and Copping, '30), vitamin B_6 (György, '35; Halliday and Evans, '37), vitamin H (Booher, '37), and the more detailed current postulates of the California, the Missouri, and the Wisconsin investigators, as to aid in the formulation of a clearer and more unified hypothesis as to the number and properties of the relatively heat stable, water soluble vitamins which function in mammalian nutrition.

SUMMARY

The primary purpose of this work was to determine the relative concentration, in the chief tissues of a presumably representative omnivorous mammal, of the nutritional factor which has been successively known as vitamin G, lactoflavin and riboflavin.

The tissues fed in the main series of experiments were from rats which had been reared on the same diet as in the case of previous studies of distribution of vitamin A. With riboflavin as with vitamin A the concentration in the liver was higher than in the skeletal muscle, and the concentrations in heart and kidney were intermediate; but the distribution of riboflavin shows a much less pronounced quantitative difference than was found in the case of vitamin A; for of vitamin A the concentration was 200 to 400 times as high in the liver as in the muscle, while of riboflavin the concentration in the liver was only ten to twenty times as high as in the skeletal muscles of the same animals. So far as may be judged from smaller numbers of measurements, the concentration of riboflavin was about tenfold higher in kidney, fivefold higher in heart, two- to threefold higher in brain and in spleen, than in skeletal muscle of the same well-nourished animals.

The level of dietary intake of riboflavin is found to influence the concentration of riboflavin in the body tissues generally.

In neither type of experiment were the differences great enough to indicate any such large storage capacity for riboflavin as the body possesses for storing vitamin A in its liver.

The bearing of these findings (and of others reported in the text) upon current concepts regarding the numbers and functions of the nutritionally essential factors of the vitamin B complex are discussed.

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CALCIUM DEFICIENCY AND INTESTINAL STASIS[1]

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FOUR FIGURES

(Received for publication July 1, 1937)

When young or half-grown rats are fed a diet deficient in minerals, their lower intestines become dilated and overloaded with fecal material and the excretion of barium or carmine is abnormally prolonged (Robertson and Doyle, '35). The addition of calcium to the mineral deficient diet almost entirely prevents this stasis, and does so completely if potassium is added as well (Robertson, '37). In addition we found that if children were fed a diet low in calcium and potassium, 75% of them became definitely constipated. We were therefore interested to see if a diet deficient in calcium alone or with an associated deficiency of potassium would lead to intestinal stasis and dilatation in rats, and to decide this question the following experiments were carried out.

The diets used in these experiments were similar to those used formerly, but the adequate diet contained 3.5% of Hawk and Oser's ('31) salt mixture, instead of Osborne and Mendel's ('18) mixture, and also the 1% NaCl was omitted. To make a diet deficient only in calcium, the calcium salts were left out of the Hawk and Oser mixture. When 1.8% of this special mixture was put in the diet, the rats received as much of the other cations as the adequate controls, but the P in the diet was reduced from 0.33% to 0.25%. Similarly the potassium salts were omitted in making the diet deficient in potassium

[1] An excerpt from a thesis submitted in conformity with the requirements for the degree of doctor of philosophy in the University of Toronto.

and in this the P was reduced to 0.21%. In a similar way the potassium and calcium salts were omitted to give a diet deficient both in potassium and calcium. In this diet no P was present in the salt mixture. As there was some Ca, K and P in the rest of the diet, all of the diets were only partially deficient in these elements.

Shortly after weaning, litters of rats were divided into five groups and fed the four diets outlined above and also the mineral deficient diet which contained the same basic

TABLE 1

Excretion of barium by rats fed various diets. Each figure is the average of from twenty to thirty-four rats

DIET	BARIUM IN FECES (DAYS)	
	X-ray determination	Chemical determination
Adequate	4.1	13
Adequate without K	3.7	14
Adequate without Ca	9.0	20
Adequate without Ca and K	10.0	21
Mineral deficient	9.0	20

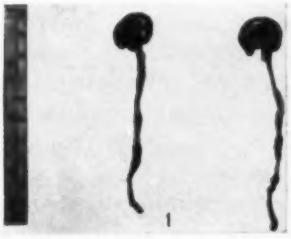
ingredients (cornstarch, casein, Crisco, dried brewer's yeast and cod liver oil) but no salt mixture. For brevity the diets were known as adequate, adequate without potassium, adequate without calcium, adequate without calcium and potassium, and mineral deficient.

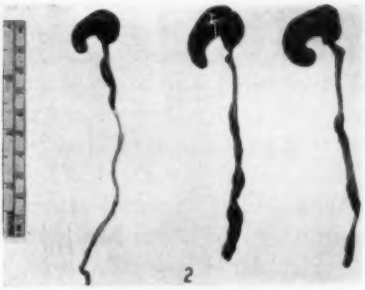
Fig. 1 Cecums and colons of rats fed adequate diet (left side) and adequate diet deficient in potassium (right side). No difference in size can be seen. The rats were of the same age and weight.

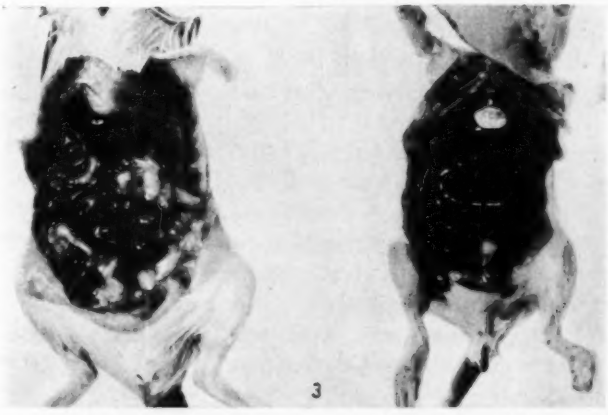
Fig. 2 Cecums and colons of rats fed the adequate diet (left side), adequate diet deficient in Ca (center), and adequate diet deficient in Ca and K (right side). Note the larger cecums and colons in the rats deficient in Ca or Ca and K. The body weights of the rats were from 106 to 110 gm.

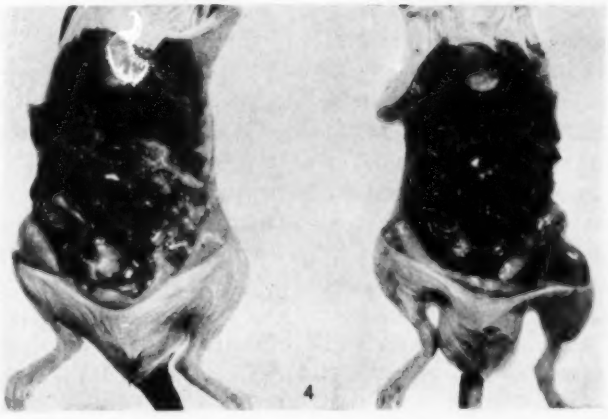
Fig. 3 Abdominal contents of rats of the same age fed adequate diet (left side), and adequate diet deficient in calcium and potassium (right side). The cecum, which is the stomach-shaped organ is much larger in the deficient animal and contains darker material. The adequately fed rat weighed 175 gm., the deficiently fed 113 gm.

Fig. 4 Abdominal contents of rats of the same age fed adequate diet (left side) and mineral deficient diet (right side). The cecum is much larger in the deficient animal, despite its much smaller body weight. The deficient rat weighed 125 gm., the adequately fed 175 gm.









After 5 to 7 weeks on the diets the rats were fed 1 dose of barium sulphate and the feces from each rat were then collected daily, and x-rayed and analyzed chemically to determine whether barium was present, using the methods described previously (Robertson, '37). As shown in table 1, the rats fed any of the diets deficient in calcium, no matter whether it was the mineral deficient, the adequate without calcium, or the adequate without calcium and potassium, all excreted the barium slowly. Possibly those fed the diet deficient in both calcium and potassium were a little slower than the others. Those fed the 'adequate without potassium' diet excreted the barium as rapidly as the adequately fed controls. In a personal communication, Prof. M. Bodansky recently informed the author that he also had observed intestinal stasis in rats fed diets low in calcium.

The rats were killed after 3 months on the diets. The intestinal tracts of the adequate controls and those fed the 'adequate without potassium' diet looked normal. The lower ileum contained only a moderate amount of material, the cecum was not enlarged and contained grayish feces and there was relatively little, gray, firm, fecal material in the colon. The cecums and colons of two typical rats are shown in figure 1.

On the contrary, in the rats fed the diets deficient in calcium there was much more material in the lower ileum and it was darker in color. The cecums were very large and contained blackish, four-smelling material and the colons were dilated and contained more dark, soft feces. These findings are shown in figures 2, 3 and 4.

In many of the rats fed the 'adequate without calcium' diet, the fur on the lower abdomen and back legs was thin and short, and many were also seen to have tetanic convulsions, especially if they were handled. About 10% of these rats died before the end of the experiment.

In further experiments the effects of these diets on the calcium, inorganic phosphorus and protein in the serum are being investigated.

CONCLUSIONS

If young rats are fed a diet low in calcium (about 0.08%) with or without an associated deficiency of potassium, they develop marked stasis and dilatation in the lower intestines, and they excrete barium sulphate very much more slowly than the adequately fed controls.

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BODY COMPOSITION AS A FACTOR GOVERNING THE BASAL HEAT PRODUCTION AND THE ENDOGENOUS NITROGEN EXCRETION

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ONE FIGURE

(Received for publication June 30, 1937)

The heat production of warm-blooded animals is generally believed to be proportional to approximately the two-thirds power of body weight. It is also commonly assumed that animals with an excess of adipose tissue have a lower basal metabolic rate (B.M.R.) per unit body weight than those containing less fatty tissue. Recent work would seem to indicate that the endogenous urinary nitrogen excretion is proportional to the basal metabolic rate. If such were the case then the same laws that govern the basal metabolic rate should likewise govern the endogenous nitrogen excretion.

Brody, Proctor and Ashworth ('34) have compiled a considerable amount of data from the literature showing the following relationships to hold for adult animals:

$$Q = 70.5\ M^{0.73}$$
$$N = 146.\ M^{0.73}$$

where Q is basal metabolism in Calories per day, M is body weight in kilograms, and N is endogenous urinary nitrogen in milligrams per day. If we disregard the difference in exponents and divide the second equation by the first we find that 2.07 mg. endogenous nitrogen are excreted for every Calorie of basal heat produced. Smuts ('35) found the above

[1] Alexander Brown Coxe Fellow, 1935–1936.

constant to be 2.004 ± 0.015. Using these values both the above papers propose to estimate the maintenance protein requirement of an animal from its basal heat production.

Whether such a relationship can be used for growing animals has been questioned by Ashworth ('35 a). Rats of weaning size were found to excrete less than 1 mg. endogenous urinary nitrogen per Calorie of basal heat produced while under the same conditions adult rats excreted 1.5 mg. nitrogen per Calorie. The object of the present investigation was to learn whether the body composition of the animal might affect independently the endogenous nitrogen excretion and the basal heat production, thus accounting for some of the variation which was found to exist in the ratio between them.

EXPERIMENTAL

Male albino rats ranging in body weight between 40 and 350 gm. were used. No attempt was made to produce animals with abnormal body compositions. We wished to determine only whether the normal variations of endogenous nitrogen excretion and basal metabolism could be accounted for by normal variations in the composition of the body. The animals used were given a diet capable of producing rapid growth (Smith and Smith, '34) from weaning until the time at which their endogenous nitrogen excretion was to be determined. They were then given a nitrogen-free diet composed of 58 parts dextrin, 10 sucrose, 4 Osborne and Mendel salts, 10 butterfat, 15 Crisco and 3 cod liver oil. The nitrogen-free diet was supplemented daily with 0.1 cc. of a tikitiki preparation containing 50 international units of vitamin B(B_1) per cubic centimeter.

After a preliminary period of 4 days on the nitrogen-free diet urine and feces were collected for 5 additional days in a collection chamber described elsewhere (Ashworth, '35 b). Following the last day of excreta collection the rats were fasted 17 to 20 hours and their basal metabolic rate measured in an open circuit Haldane apparatus. Two trains of absorption tubes were used so one set could be weighed while the

other was used to ventilate the animal chamber. CO_2 production was measured for six periods ranging in length from 30 to 70 minutes depending on the size of the animal. The mean of the three lowest values for CO_2 production together with the R.Q. over the entire period of 4 to 6 hours was used to calculate the basal heat production.

Immediately after completing the basal metabolic rate measurement the rats were killed, hashed and dried in a vacuum oven at 70° until there was no further loss of weight. The carcasses were then partially extracted with ether, ground in a mortar and passed through a 1 mm. sieve. Samples were weighed out for the determination of nitrogen (macro Kjeldahl, using the boric acid modification) and fat (Light, Smith, Smith and Anderson, '34).

DISCUSSION OF THE RESULTS

The original data are shown in table 1. It will be observed that there is considerable difference between the initial and final body weights of these animals. The initial body weights were recorded just before the animals were put on the nitrogen-free diet. While subsisting on such a diet prevention of body weight loss was not possible. This may be partially due to the fact that the rats had been accustomed to receiving a diet high in protein. Insufficient caloric intake is a criticism often made of similar results. We have kept food intake records to see whether the energy intake was large enough to prevent the loss of body protein for energy purposes.

It is difficult to estimate just what are the optimal caloric requirements of these animals. The small rat needs a proportionately larger excess above the basal for muscular activity than does the older, more sedate rat because, with an ad lib. intake of nitrogen-free food, the ratio of food Calories in excess of basal to basal Calories varied between 1.5 and 2.6 for rats weighing less than 100 gm., while it varied between 0.5 and 1.5 for larger rats. The minimal value of the above ratio indicates a caloric intake 50% greater than the basal metabolic rate requirement and would seem to be suffi-

cient for rats confined to small cages. No satisfactory demonstration could be made of a correlation between loss in body weight and the amount of nitrogen-free diet consumed although the results would indicate that the rats consuming the larger amounts of nitrogen-free diet, above a certain optimal level, lost weight more rapidly, a finding contrary to expectation.

TABLE 1

Original data

RAT NO.	BODY WEIGHT		AVERAGE FOOD INTAKE[1]	BASAL HEAT	NITROGEN		BODY COMPOSITION		
	Initial	Final			Urine	Feces	Dry weight	Total fat	Total N
	gm.	*gm.*	*gm./day*	*cal./day*	*mg./day*	*mg./day*	*gm.*	*gm.*	*gm.*
11	88	68	3.8	11.0	26.0	7.6	23.6	9.0	2.20
12	99	75	4.2	11.8	27.4	9.1	25.4	9.5	2.15
13	131	96	4.5	15.9	40.2	7.6	32.6	10.6	2.98
15	143	110	7.4	18.8	35.2	12.3	42.6	19.2	3.12
14	129	106	5.5	15.8	30.8	9.9	41.7	18.2	3.35
16	146	121	7.7	19.1	30.8	14.6	49.7	23.4	3.68
21	46	34	4.0	6.8	11.6	...	11.0	4.4	0.89
31	62	46	4.4	9.3	14.7	6.9	15.2	5.0	1.39
22	55	47	4.7	8.7	12.5	8.7	15.8	6.6	1.24
32	61	50	5.0	8.2	15.3	7.9	16.1	5.6	1.43
41	91	68	4.7	6.5	23.5	9.7	21.3	6.5	2.10
51	95	68	3.3	7.0	28.7	6.6	22.0	7.0	2.02
23	330	277	12.7	27.2	72.8	24.9	115.7	55.4	9.11
42	350	295	10.7	28.4	78.4	22.3	112.0	42.6	9.49
43	340	283	11.0	30.5	82.8	23.3	98.6	30.6	9.59
81	323	269	10.3	24.0	59.8	21.4	114.0	54.4	8.45
83	332	280	9.0	27.9	74.2	21.8	115.7	56.8	8.46
84	317	264	10.8	27.0	68.4	22.7	106.7	49.1	8.29
100	135	117	5.4	14.9	40.0	...	41.5	14.8	3.55
101	175	153	8.0	23.2	46.8	...	58.0	22.8	4.83

[1] Food intake during urine collection.

Since the rats were losing body weight at the rate of about 1% per day, it was considered advisable to determine the endogenous nitrogen excretion before the absolute minimal level was reached. There are many who believe that a preliminary period of 4 days on a nitrogen-free diet is all that is necessary to reduce the excretion of nitrogen to the minimum level in the rat. Ashworth and Brody ('33) have demonstrated that under the conditions here used rats do not reach their minimum level of nitrogen excretion in that length of

time. Smuts ('35) suggested that these rats may be excreting abnormally low amounts of nitrogen.

The probability that long periods of nitrogen-free feeding would produce changes in the body composition of the rat was considered likely. Such changes would be difficult to interpret when attempting to make any correlations with the minimal energy and nitrogen outputs of the animal. Therefore in this study the rats were kept on the nitrogen-free diet only 4 days preliminary to the collection of excreta.

TABLE 2

(1) log Cal = 0.648 log live wt. — 0.126
S.E. = 0.041 r = 0.979

(2) log Ur N = 0.853 log live wt. — 0.208
S.E. = 0.057 r = 0.976

(3) log Cal = 0.581 log dry body wt. + 0.266
S.E. = 0.042 r = 0.978

(4) log Ur N = 0.757 log dry body wt. + 0.321
S.E. = 0.072 r = 0.962

(5) log Cal = 0.622 log fat-free dry wt. + 0.338
S.E. = 0.039 r = 0.981

(6) log Ur N = 0.820 log fat-free dry wt. + 0.400
S.E. = 0.051 r = 0.981

(7) log Cal = 0.606 log body N + 0.880
S.E. = 0.041 r = 0.979

(8) log Ur N = 0.800 log body N + 1.114
S.E. = 0.054 r = 0.979

(9) log Cal = 0.734 log Ur N + 0.073
S.E. = 0.050 r = 0.968

Since surface area is commonly related to a fractional power of body weight and used as a unit of reference for the basal metabolic rate, power equations were used in the present investigation to correlate both basal metabolism and endogenous nitrogen with live body weight and with the various components of the body such as dry matter, fat-free dry weight and nitrogen content. Table 2 is a summary of these power equations in their logarithmic form together with their statistical constants. The equations were fitted by the method

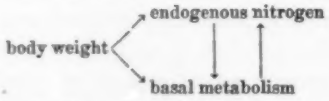
of least squares, the procedure being one of those outlined by Davenport and Ekas ('36). The wide range of body weight used probably accounts for the high correlation coefficients. The standard errors are, of course, in logarithm units.

One may readily observe that the components body nitrogen and fat-free dry weight offer the best standards of reference for both endogenous nitrogen and basal energy metabolisms.

No reason is apparent for the relatively low value for the correlation coefficient between endogenous nitrogen and the dry body weight of the animals (equation no. 4). When the body fat is removed there is a significant increase in the correlation coefficient as one may observe from equation no. 6.

If there were some fundamentally constant relationship between the endogenous urinary nitrogen excretion and the basal energy metabolism one would expect a higher correlation between them than between either of them and the various components of the body. Such is not the case. The correlation coefficient associated with equation no. 9 is, with the exception of that belonging with equation no. 4, the poorest of all. This fact would lead one to believe that endogenous nitrogen excretion and basal energy output are not proportional to each other.

In order to be entirely sure of this we have utilized the concept of partial correlation. Such a procedure is used when it is desired to eliminate some factor which may be affecting the correlation between the two factors under consideration. For example one may postulate that body weight is contributing to the correlation between endogenous nitrogen and basal metabolism as illustrated in the following diagram.



Partial correlation is then used to eliminate the effect of body weight. The formula used was as follows: (taken from Davenport and Ekas, '36; p. 109)

$$r_{12.3} = \frac{r_{12} - r_{13} \cdot r_{23}}{\sqrt{1 - r^2_{13}} \sqrt{1 - r^2_{23}}}$$

where the subscripts stand for the factors correlated. In the above example these are 1) endogenous nitrogen, 2) basal metabolism, 3) body weight.

The value of the partial correlation for the example given above, i.e., the correlation between endogenous nitrogen and basal metabolism holding the effect of the final live weight constant, was 0.27 ± 0.13 which is not significantly different from zero when its probable error is taken into consideration. Likewise when the effect of the various body components are eliminated by partial correlation the net correlation between endogenous nitrogen and basal metabolism remains without significance.

By supplementing the data of table 1 with those taken from a previously published paper (Ashworth, '35 a) the partial correlation of endogenous urinary nitrogen with basal metabolism holding live weight constant could be studied in more detail. Values for the original correlation coefficients were

basal Calories: body weight—0.946
endogenous nitrogen: body weight—0.939
basal Calories: endogenous nitrogen—0.871

The correlation of heat production with endogenous nitrogen is significantly less than the correlation of either with body weight. When this latter correlation is corrected for the effect of body weight by partial correlation the resulting net value is —0.16 which shows a tendency toward an inverse relationship except that it is not large enough to be significant for the number of animals used (130 in this case).

Exponential equations for the first two of the above correlations were found to be:

$$\text{Calories} = 0.540 \text{ body weight}^{0.739}$$
$$\text{endogenous nitrogen} = 0.272 \text{ body weight}^{0.982}$$

The large difference in exponents is quite significant in showing the absence of parallelism between the basal heat production and the endogenous nitrogen excretion of the growing rat. Similar differences are noted in table 2.

When the basal heat production of these animals is plotted directly against the endogenous urinary nitrogen excretion,

the curve instead of being linear in character is sigmoid. The shape of the curve is very similar to that of a typical body weight growth curve, or that of an autocatalytic reaction. Another way of presenting the same relationship is shown in figure 1. There the ratios of endogenous nitrogen excretion to basal heat production are plotted against the body weights of the animals. In order to make the trend more evident it has been smoothed out by the method of moving averages. The dotted line indicates how the trend should appear if 2 mg.

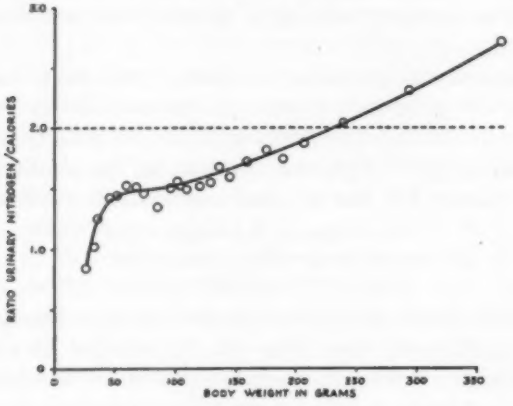


Figure 1

of endogenous nitrogen were excreted for every Calorie of basal heat produced, the thesis advanced by previous investigators. It is evident from figure 1 that there is no proportionality between the minimum requirement for protein and for energy in the growing rat.

In conclusion it appears then that the endogenous urinary nitrogen excretion and the basal energy metabolism of growing rats are not proportional to each other and that variations in body composition do not account for this lack of proportionality.

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THE ARREST OF NUTRITIONAL CATARACT BY THE USE OF RIBOFLAVIN [1]

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TWO FIGURES

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A recent report from this laboratory (Day, Darby and Langston, '37) described experiments which showed that the cataract which results from 'vitamin G' deficiency may be prevented by means of pure riboflavin,[2] either natural or synthetic. Since that report was written, rats have been kept for more than a year on a flavin-deficient diet, supplemented with 90 or 120 micrograms of riboflavin weekly, without showing cataract, keratitis or alopecia. Those animals have reached maturity, although growth proceeded at a somewhat subnormal rate on such levels of riboflavin feeding. Those experiments would seem to prove the identity of the rat cataract-preventive vitamin with flavin. The final test of this identity, however, would be experiments planned to show whether or not the development of cataract could be arrested by administration of pure riboflavin. This paper reports such experiments.

[1] Research paper no. 513, journal series, University of Arkansas. Read before the Division of Biological Chemistry of the American Chemical Society in Chapel Hill, April 13, 1937.

[2] The term riboflavin is used in this paper to mean the substance described as 6, 7-dimethyl-9-(*d*-1'-ribityl)-isoalloxazin (lactoflavin), in conformity with the terminology accepted by the Council on Pharmacy and Chemistry of the American Medical Association ('37).

In order to prevent confusion, it should be stated here that cataract in rats resulting from flavin deficiency is apparently quite different in etiology from the cataract which results from lactose or galactose feeding (Mitchell, Merriam and Cook, '37). The two types of cataract may be distinguished with the ophthalmoscope in the hands of a skilled observer. One type must be regarded as due to the deficiency of an essential substance; the other apparently results from the presence of a substance which, in high concentrations in the rat body, is harmful to lens tissue.

EXPERIMENTAL PROCEDURE

Our flavin-deficient diet, method of caging and care of animals, stock diet, and experimental procedure in flavin experiments with rats have been repeatedly described (Day, Darby and Langston ('37) and references cited therein). Two features of our technic might be pointed out as probably being especially important, however. The casein and other constituents of our experimental diet are flavin-free according to the most rigid tests available at the present time. The young rats are started on experiment when they are 21 days of age, at which time they weigh between 30 and 45 gm. each.

In the series of experiments to be reported in this paper, forty-three rats from six litters were given the flavin-deficient diet (no. 625). Weekly weight and food consumption records were kept, and the eyes were examined with an ophthalmoscope at weekly intervals or oftener. When early cataractous changes appeared in the eyes of the rats, certain of the animals from each litter were given riboflavin, while others were retained as controls.

The synthetic riboflavin used in this experiment [3] was supplied to us in sterile ampules, each containing 1 mg. of riboflavin in 2 cc. of solution. Preliminary experiments indicated that it was sometimes difficult to get the deficient rats to consume the solution quantitatively. Consequently, in the experiments here reported, the flavin solution was administered by injection into the leg muscles. Each animal received

[3] Supplied by the Winthrop Chemical Company.

0.24 cc. of the solution, equivalent to 120 micrograms of riboflavin, twice weekly. Twenty-five of the forty-three rats were given injections of riboflavin.

Although attention has previously been called to this fact, it is important to point out here that the cataractous changes in the lens of the eye in flavin deficiency are usually accompanied by, and sometimes preceded by, a generalized ophthalmia and dense opacities in the cornea (keratitis). This ophthalmia and keratitis make it sometimes difficult to see opacities in the lens (cataract) in their early stages. It is obvious that if administration of flavin were deferred until unmistakable evidence of cataract appeared—that is, until mature cataract developed—it would be futile to attempt to arrest the progress of the lens changes. Consequently, in our attempt to start the administration of flavin while cataract development was in its incipient stages, some errors of judgement were made. In some cases the flavin injections were started so late that the cataract went on to maturity. In other cases it was found that, after the animal had resumed growth and the keratitis cleared, no lens opacities were present. This was conceivably due to a 'cure' of cataract—a regression of the lens opacities. It is more probable, however, that no cataract had ever been present.

RESULTS AND DISCUSSION

Eighteen control animals received no supplement of riboflavin. Two of of these animals died during the first few weeks of experiment (twenty-ninth and thirty-second days) without exhibiting cataract. Of the remaining sixteen control animals, thirteen (81%) exhibited cataract, and the average time of appearance was 52 days. The cataract proceeded to maturity in twelve rats (75%), at an average time of 67 days. It is therefore apparent that in this series of rats, as in similar groups previously reported from this laboratory, cataract appeared in a high percentage of those animals receiving an unsupplemented flavin-deficient diet, and the cataract proceeded to maturity in almost all such animals. The average survival of the sixteen control rats was 74 days.

Table 1 gives a summary of the data concerning the animals given riboflavin. Twenty-five rats were in this group, and for the reasons explained above, it was found as the experiment progressed that six of the rats did not have cataract. Of the nineteen rats showing lens changes, cataract proceeded to maturity in both eyes in two rats. By 'maturity' we mean opacities so marked that the eye appeared white by the most casual observation, and without the use of an ophthalmoscope or other instrument. In each of six other rats the cataract proceeded to maturity in one eye, but its progress was definitely arrested in the other eye. Cataract was arrested in

TABLE 1

Data on the arrest of cataract in rats given intramuscular injections of 120 micrograms of riboflavin twice weekly after early cataractous changes had appeared

	NUMBER	PER CENT OF TOTAL	PER CENT OF THOSE HAVING CATARACT
Arrested, both eyes	11	44	57.9
Arrested, one eye	6	24	31.6
Total arrested	(17)	(68)	(89.5)
Not arrested	2	8	10.5
Both eyes clear	6	24	
Total	25	100	100.

both eyes of the remaining eleven rats. Cataract was therefore definitely arrested in one or both eyes of seventeen out of nineteen rats with cataract (89.5%), and proceeded to maturity in both eyes of two of the nineteen rats (10.5%). In the rats with arrested cataract, no change in the condition of the lens can be seen now after 4 months.

Upon the administration of flavin there was an immediate and pronounced growth response (fig. 1). Where alopecia had been evident, new hair appeared. Ophthalmia cleared up rapidly, and keratitis eventually disappeared. During the period of rapid growth scabby ulcers appeared upon the head and body of many of the rats. The exact significance of this is obscure, but we have observed the same phenomenon whenever a source of flavin (as yeast) has been given rats which had

been deprived of the vitamin for an extended period. It appears to be a response to the feeding of flavin, and not an evidence that a deficiency of another substance has been superimposed.

The condition in the lenses with arrested cataract took several forms. In a number of eyes the entire lens was found to be clear except for a patch of opacity near the periphery

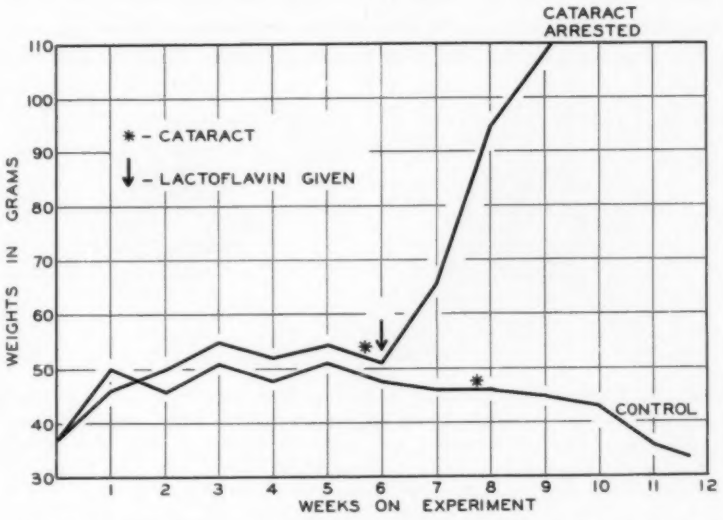

Fig. 1 Weight curves of typical experimental animals. After early cataractous changes were seen in the eyes, 120 micrograms of riboflavin were given twice weekly to certain animals. Control animals were kept on the flavin-deficient diet until death.

of the lens, frequently in the side toward the ear. In other eyes it took the form of diffuse opacities throughout the lens. As seen through the ophthalmoscope these would frequently look like shattered 'shatterproof' glass. Such eyes would appear quite normal to the unaided eye in normal illumination. However, if carefully observed in a darkened room by the light of a small pocket flashlight, a snowy mass could be seen in the eye.

In one or both eyes of certain animals the arrested cataract took a third form. The lens opacities proceeded to maturity in the center of the lens, with a zone of less dense opacities surrounding this nuclear cataract, and a zone of clear lens tissue at the periphery of the lens. Figure 2 shows an enlarged photograph of a normal eye, and of an eye exhibiting arrested cataract of this type. Although it is only conjecture, there appears to be a very simple and reasonable explanation for this. In these cases of nuclear cataract with clear surrounding lens tissue, the central cataractous portion is about

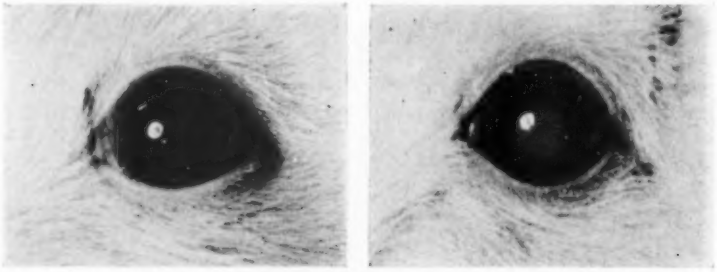

Fig. 2 Enlarged photographs of eyes of two rats. Left, normal eye. Right, cataract arrested with riboflavin; in the lower center of the lens mature cataract (white) is evident. Surrounding this mature cataract is a zone of less dense opacities, and outside of this zone clear lens tissue is seen. The highlights on the eyeball are reflections from the flash-lamp and should be ignored.

the size that the entire lens was at the time that flavin administration was started. Since there is no mechanism for the repair or replacement of badly damaged lens fibers, cataract existing at the time of flavin administration would persist as a mass of opacities in the center of the lens. If we postulate that in these eyes the lens epithelium had not been destroyed, it would be possible, upon growth of the lens induced by flavin administration, for the lens epithelium to lay down normal lens fibers around this cataractous tissue. Such is apparently what happed in a number of cases. The cases of arrested cataract with diffuse opacities throughout the lens are less easily explained.

In 1934 we reported that nutritional cataract had been arrested by the feeding of milk powder to rats with early cataractous changes (Langston and Day, '34), and the action of the milk powder was ascribed to its 'vitamin G' content. It is now possible to reinterpret those data in terms of the riboflavin contained in the milk powder.

From the experiments reported in this and foregoing papers from this laboratory (Day, Darby and Langston, '37), it is now possible to conclude quite definitely that, with our diet and selection and care of experimental animals (rats), flavin deficiency results in cataract in a large percentage of cases. Furthermore, such cataract can be prevented by the feeding of pure riboflavin, and the progress of cataractous changes can be arrested by the timely administration of riboflavin to rats with incipient cataract. These experiments furnish clear evidence that riboflavin is a specific cataract-preventive substance for the rat.

SUMMARY

Young albino rats were given a diet deficient in flavin and their eyes were examined at frequent intervals with the ophthalmoscope. Of sixteen control animals receiving the deficient diet without supplement, thirteen (81%) developed cataract at an average time of 52 days. The cataract proceeded to maturity in twelve (75%) of these rats, at an average time of 67 days. The average survival was 74 days.

When early cataractous changes were evident, twenty-five rats were given intramuscular injections of riboflavin in doses of 120 micrograms twice weekly. The animals rapidly increased in weight, new hair appeared on those rats with alopecia, and keratitis slowly cleared up. In eleven of these rats cataract was arrested in both eyes. In each of six other rats the cataract proceeded to maturity in one eye, but its progress was definitely arrested in the other eye. The cataract proceeded to maturity in both eyes of two rats. Six rats were found to have clear lenses in both eyes after the keratitis cleared up. It is thus apparent that the progress of cataract

development was arrested by riboflavin administration in seventeen out of nineteen animals exhibiting cataract (89.5%). These data furnish additional evidence that flavin is the cataract-preventive vitamin.

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THE INFLUENCE OF SEX ON IRON UTILIZATION IN RATS [1]

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ONE FIGURE

(Received for publication July 6, 1937)

The possibility of a sex difference in utilization of iron was suggested first by Mitchell ('32) and very soon afterward by Rose and Kung ('32). Mitchell observed that at weaning time there was a slightly higher hemoglobin concentration in the blood of male than of female rats. She had records of 283 males having an average hemoglobin of 8.2 gm. per 100 cc. of blood and of 291 females averaging 9.2 gm. She found also that the average length of time required to induce severe anemia was 1.1 weeks longer for a group of 133 females than for the corresponding group of 129 male litter mates. Rose and Kung ('32) found that to induce anemia by removal of blood was more difficult in case of females than of males. In a group of twenty-three females and fifty-five males, there were twenty-two animals from which more than the average amount of iron had to be removed by bleeding to accomplish their purpose and of these eighteen were females. These studies raised the question as to whether there was some characteristic of the female which caused her to utilize iron more economically than the male. Mitchell suggested that a better prenatal store of iron might explain the tendency of females to become anemic more slowly than the males on

[1] Based on a thesis presented by Helen Jackson Hubbell in partial fulfillment of the requirements for the degree of doctor of philosophy.

a diet poor in iron. However, Rose and Kung, having carried their animals to a normal hemoglobin level and then depleted them again, thought the observed differences might be accounted for by the greater rate of growth of the male and the larger food consumption in consequence. Smith and Otis ('37) have, since this work was completed, published a study of equal numbers of male and female rats fed the same amount of iron at the end of a depletion period and followed for 6 weeks thereafter to observe any differences in their gains in hemoglobin. On various levels of iron intake up to 0.2 mg. daily, the females produced slightly more hemoglobin than the males. In their study, however, the iron intake of the females was not kept the same per gram of rat as that of the males.

There are but few analyses of the iron content of the bodies of normal rats reported in the literature. Smythe and Miller ('29) give the most complete data for different age groups but the number of females used is too small to determine averages for the two sexes separately.

The aim of the present investigation has been to study the utilization of iron after ruling out the influence of the inherent sex difference in growth by feeding iron and copper in dosages carefully adjusted to changing body weights throughout the whole period of investigation. The general plan was to reduce young male and female rats to a uniform state of anemia by milk feeding, then to feed the depleted animals iron and copper supplements, the dosage of iron for each rat being calculated for each week on the basis of its body weight, and that of copper being sufficient to insure full utilization of the iron for hemoglobin formation. When the hemoglobin was practically uniform at 14 gm. per 100 cc. of blood the bodies were analyzed for iron and sexes compared as to the percentage of iron found.

METHODS

Well-matched male and female rats 21 to 27 days old, weaned from mothers fed Sherman's diet B, consisting of one-third whole milk powder and two-thirds whole wheat plus sodium

chloride and supplemented by lean beef, were placed in separate galvanized iron wire cages freshly treated with aluminum paint, having raised bottoms of ½ inch mesh to prevent consumption of feces. All animals were weighed weekly and their consumption of milk was recorded. Quantitative hemoglobin determinations were made by the colorimetric method described by Exton and Rose ('33) using the Exton photoelectric scopometer ('32). Duplicate samples of 0.025 cc. of blood drawn from snipped tails were placed in 5 cc. of tenth normal hydrochloric acid and after standing 20 to 45 minutes were read in the scopometer.

When the animals had been depleted to between 4 and 5 gm. of hemoglobin per 100 cc. of blood, some were killed and reserved for iron analyses and the rest were fed dried whole milk supplemented by iron and copper, the amount of iron being adjusted weekly according to the body weight of each animal. During this period, hemoglobin determinations were made from the fourth week of iron and copper feeding until the hemoglobin reached approximately 14 gm. per 100 cc. of blood when they were killed and their bodies analyzed for iron. Most of the animals reached this level within 6 weeks. A few which did not were kept on the iron feeding through the seventh week. All of them reached a level of more than 13 gm. per 100 cc. of blood.

The bodies, carefully ashed as described by Rose and Kung ('32) were analyzed by the method of Rose, McCarthy, Blacker, Schattner and Exton ('36). The ash of each rat body was dissolved in hydrochloric acid and made up to 100 cc. so that the acidity of the final solution would be about 10%. For each determination 0.5 or 1 cc. of rat solution was placed in a small test tube and 3 cc. of water and 0.5 cc. of a normal solution of potassium thiocyanate were added. Three successive extractions of the resulting ferric thiocyanate were made with iron-free amyl acetate in portions of 4 cc., 3 cc. and 3 cc., gently tipping the test tube back and forth fifty times after each addition and then pouring the contents into a 30 cc. pear-shaped separatory funnel by means of which the aqueous layer

was withdrawn for the next extraction. The amyl acetate solution, collected in a 15 cc. centrifuge tube, was centrifuged for 1½ minutes to remove the last trace of turbidity before readings were made on the scopometer. Determinations were always made at least in quadruplicate and independent analyses were made by each of us. The delicacy of the method is indicated by some typical figures shown in table 1.

Since in some methods the reactivity of the iron is interfered with by the formation of pyrophosphates, a careful study

TABLE 1

Some iron analyses of rat bodies made at different times and in part by two analysts

ANALYST	RAT NO.	DATE	SCOPOMETER READING	MILLIGRAMS Fe PER RAT
H	XI M_2	10/5	408	6.66
			410	6.75
		10/15	407	6.63
			408	6.66
H	XI F_2	10/15	273	0.77
			273	0.77
		10/16	274	0.78
			275	0.80
H	II M_2	3/22	343	4.73
			345	4.76
		10/12	345	4.76
			343	4.73
H	VII F_1	9/28	395	6.26
			393	6.20
R	VII F_1	10/24	395	6.26
			395	6.26
H	IV M_2	9/18	399	6.36
			399	6.36
R	IV M_2	10/23	400	6.40
			398	6.33
H	III F_1	10/8	291	1.76
			290	1.74
R	III F_1	10/9	290	1.74
			291	1.76

was made to be sure that there was no such interference in applying this method to rat bodies. A number of samples of rat ash solutions tested before and after boiling with hydrochloric acid for 1 hour with a reflux condenser gave practically identical results. As a further test pyrophosphate was added to one of duplicate samples of known iron content and both were then subjected to the same ashing process as the rat bodies and analyzed for iron. The results were in as close agreement as the quadruplicate analyses on either sample thus showing conclusively that under the conditions described there is no interference of pyrophosphates.

Some checks of the scopometer method were made by the Zimmermann-Reinhardt method, which has been used with success in this laboratory but which was not used in this study because it requires too large samples to permit more than one or two tests on the same rat body, whereas with the scopometer a dozen were sometimes made and fifty or more would have been possible. Analyses of four rat bodies agreed within 2 to 4% which is within the experimental error of the Zimmermann-Reinhardt method. In four other comparisons, the differences were slightly higher, but the average of the eight samples was a difference of 4.3%, and it must be borne in mind that only one sample was available for each Zimmermann-Reinhardt determination.

In case of milk, it was necessary to modify the method to avoid the interference resulting from the high concentration of salts, especially phosphates, in the solution obtained by dissolving the ash of 40 gm. of powdered milk or 300 gm. of fresh milk in hydrochloric acid and making it up to 50 cc. It was necessary to take 5 cc. of this solution for a determination. The acidity (already 12 to 15%) was increased by adding 2 cc. of concentrated hydrochloric acid, and the ferric chloride in solution was extracted three times with a mixture consisting each time of 5 cc. of ether and 1 cc. of acetone. A fourth extraction, tested with potassium thiocyanate, was always found to be iron-free and was discarded. These extracts were combined in a 25 cc. beaker and the ether and

acetone evaporated over warm water in a dust-free alberene hood in a protected inner room, under a tent of filter paper. Then 2 cc. of water was added to the residue, the solution was made slightly alkaline with a few drops of twice normal sodium hydroxide, neutralized with hydrochloric acid, using phenolphthalein as indicator, and again acidified with 2 drops of concentrated hydrochloric acid. Thereupon 0.5 cc. of normal potassium thiocyanate was added and extraction made with amyl acetate as already described.

The iron and copper were fed to the rats as ferric chloride and copper sulphate, respectively, the solutions being so adjusted that 1 cc. of ferric chloride solution contained 0.4 mg. of iron, and 1 cc. of the copper sulphate solution contained 0.1 mg. of copper. On weighing days the weekly allowance was calculated for each animal and from this total was subtracted the amount of iron and estimated copper consumed in the milk during the previous week. The supplements were fed three times a week. Each dosage was given in 5 cc. of reconstituted powdered milk.

THE DEPLETION PERIOD

Since the depletion period on powdered milk was found to average 32 days, an attempt was made to hasten matters by using fluid Guernsey milk obtained by direct milking into specially cleansed glass bottles. It was thought that the high fat content (5%) of the Guernsey milk might result in a higher calorie intake and hence more rapid gain in body weight. The fluid milk had no effect on the males and caused only a slightly more rapid decrease in the percentage of hemoglobin in the blood of the females. For twenty-two males, eleven in each group, the average length of the depletion period was the same, 28 days. The average time for twelve females on powdered milk was 36 days and for fifteen females on fluid milk, 31 days. The average daily food intake in dry weight per gram of rat was 0.065 gm. for the males on powdered milk and 0.046 gm. for the males on fluid milk. For the females it was 0.070 gm. per gram of rat on powdered milk

and 0.052 gm. on fluid milk. The average hemoglobin for the eleven males in the powdered milk group was 4.3 gm. per 100 cc. of blood and in the fluid milk group, 4.0 gm. In the case of the females, the group on powdered milk averaged 4.6 gm. and the group on fluid milk 4.3 gm.

Determination of the iron content of the bodies of seven males and ten females depleted by milk feeding to from 3.4 to 4.9 gm. of hemoglobin per 100 cc. of blood gave the same results for both sexes, 0.013 ± 0.0004 mg. per gram of body weight. Since the animals which were fed iron, were depleted to the same level, namely under the same conditions, it seems safe to conclude that they, too, had a like iron content at the end of the depletion period. This is in harmony with the findings of Rose and Kung ('32) who found that animals depleted to between 5 and 6 gm. of hemoglobin per 100 cc. of blood had an average iron content of $0.0021 \pm 0.00003\%$.

Schultze and Elvehjem ('33) have called attention to the fact that if copper be given to anemic rats it will tend to stimulate the utilization of any reserve iron available for hemoglobin regeneration and so increase the hemoglobin of the blood. To make certain that under the conditions of this investigation all reserve iron was exhausted when the iron-feeding period began, 0.1 mg. of copper was fed daily to two litters of depleted animals. Of sixteen animals so tested, there were five males and two females that showed practically no change in hemoglobin after 1 week; two males and three females showed an increase of from 0.4 to 1.4 gm. per 100 cc. of blood during the first week but returned to the former level the next week; one male and three females showed increases in hemoglobin from 0.8 to 1.3 gm. per 100 cc. of blood during the first week, and remained at that level until chloroformed 3 weeks later. These very slight increases, being within normal variability, did not indicate any necessity for administration of copper during the depletion period to insure full exhaustion of iron reserves.

THE IRON-FEEDING PERIOD

There are no data in the literature regarding the iron requirement of rats on the basis of body weight, and therefore, it seemed advisable to make this study on three levels of iron intake, e.g., 0.0019, 0.0038 and 0.0057 mg. per gram of body weight per day. The copper intake was uniform for the three series, 0.00038 mg. per gram of rat per day.

In series A, the group on the lowest iron allowance, the total daily intake of iron per rat amounted to 0.14 to 0.28 mg. in the first week and to 0.26 mg. to 0.48 mg. in the last week. The copper ranged from 0.027 mg. to 0.056 mg. per rat per day for the first week. By the sixth week no animal was getting less than 0.045 mg. per day which more than covered the requirement as indicated by studies of Myers and Beard ('31) and of Hart, Steenbock, Waddell and Elvehjem ('28). In series B the minimum iron intake per rat per day ranged from 0.27 to 0.41 mg. the first week, and from 0.54 to 1.00 mg. the last week. The copper dosage was the same as for series A. In series C, the minimum iron intake per rat per day ranged from 0.41 mg. to 0.57 mg. in the first week and from 0.85 mg. to 1.64 mg. in the last week.

Analysis of the bodies when the animals had reached a hemoglobin value of approximately 14 gm. per 100 cc. of blood showed that in each series the amount of iron per gram of body weight was higher for the females than for the males. Of those in series A receiving 0.0019 mg. of iron per gram of rat per day, the seven males averaged 0.030 ± 0.0003 mg. per gram of rat and the eight females 0.034 ± 0.0005 mg.; of those in series B receiving 0.0038 mg. of iron, the eight males averaged 0.034 ± 0.0006 mg. and the nine females 0.038 ± 0.0007 mg.; of those in series C receiving 0.0057 mg. of iron, the four males averaged 0.033 mg. and the four females 0.037 mg. The differences are shown in figure 1 and the full data are given in table 2. In series A the difference between the means of the iron content of the males and females was 6.7 times the probable error of the difference; and in series B it was 4.4 times the probable error of the difference. The third series was not continued beyond the

study of eight animals because the differences between the sexes were in close agreement with those on 0.0038 mg. of iron. These differences between the sexes, expressed in percentage were 13, 12 and 11%, respectively, for the three series.

Even in the depletion period there was evidence that the females had greater reserves of iron than the males. In a group of sixteen rats equally divided as to sexes, there were three females but only one male failing to deplete to a hemoglobin level below 5 gm. per 100 cc. of blood in 7 weeks. Also the depletion periods of the females in the first two series

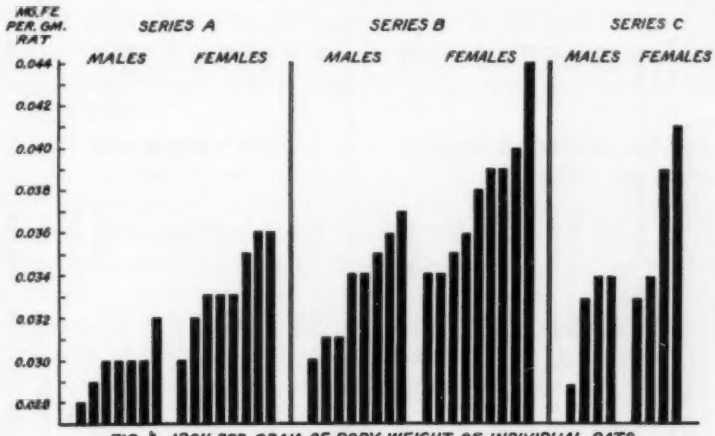

FIG. 1. IRON PER GRAM OF BODY WEIGHT OF INDIVIDUAL RATS

and in the group of anemic controls were, respectively, 24, 17 and 17% longer than those of the males.

Since there is, as shown by this study, a slight but consistent difference in the iron content of the bodies of male and female albino rats studied at different levels of intake, the question arises whether the female differs from the male in the method of utilizing the retained iron. Does the female store iron in the liver and other tissues to a greater extent than the male or does she produce more hemoglobin per gram of body weight? According to Elvehjem and others ('32), Josephs ('32), Cook and Spilles ('31) and Cunningham ('31) any absorbed iron,

TABLE 2

Weight and hemoglobin of rats at end of iron-feeding period and iron content of bodies

MALES						FEMALES					
Iron-feeding period				Iron content of rat		Iron-feeding period				Iron content of rat	
Number of days	Weight at end	Hemoglobin at end	Gain in hemoglobin	Total	Per gram rat	Number of days	Weight at end	Hemoglobin at end	Gain in hemoglobin	Total	Per gram rat
	gm.	*gm.*	*gm.*	*mg.*	*mg.*		*gm.*	*gm.*	*gm.*	*mg.*	*mg.*
Series A. Rats fed 0.0019 mg. Fe per gram rat per day											
49	245	14.8	10.2	7.41	0.032	49	183	13.7	8.8	6.36	0.036
42	265	14.9	9.9	7.61	0.030	49	165	14.0	9.5	5.07	0.033
49	233	13.5	9.6	6.26	0.028	49	191	13.8	8.5	6.33	0.035
49	221	14.6	10.8	6.35	0.030	49	154	13.4	9.1	4.75	0.033
35	187	14.3	10.0	5.34	0.030	49	154	14.2	9.3	4.94	0.033
42	223	14.2	9.7	6.29	0.030	48	178	13.2	8.7	5.43	0.032
48	241	13.7	9.8	6.95	0.029	48	182	13.7	9.2	5.41	0.030
						48	146	14.5	9.8	5.01	0.036
45	230	14.3	10.0		0.030 ± 0.0003	49	169	13.8	9.1		0.034 ± 0.0005
Series B. Rats fed 0.0038 mg. Fe per gram rat per day											
35	226	14.2	10.0	7.26	0.034	42	140	14.5	10.5	5.18	0.039
35	230	14.3	10.2	6.72	0.031	42	160	14.9	9.9	5.29	0.034
42	281	14.2	10.5	8.50	0.031	35	151	14.5	9.3	5.12	0.035
35	226	14.0	9.4	7.24	0.034	35	179	14.9	10.3	6.93	0.040
49	233	13.5	9.5	8.13	0.036	48	170	13.4	9.3	5.55	0.034
35	195	14.3	10.7	6.49	0.035	35	161	14.6	10.1	5.54	0.036
35	211	14.6	10.4	7.48	0.037	34	149	14.8	10.7	6.26	0.044
42	241	14.3	9.8	7.00	0.030	35	171	14.0	10.2	6.27	0.038
						35	152	14.9	10.1	5.65	0.039
39	230	14.1	10.0		0.034 ± 0.0006	38	159	14.4	10.0		0.038 ± 0.0007
Series C. Rats fed 0.0057 mg. Fe per gram rat per day											
42	272	15.0	11.8	8.56	0.033	42	160	14.5	10.4	5.24	0.034
42	222	12.5	8.5	6.03	0.029	41	175	13.3	9.0	5.55	0.033
40	217	14.1	10.1	6.97	0.034	42	149	13.5	9.9	5.83	0.041
42	200	14.7	10.6	6.69	0.034	41	173	13.9	9.0	6.47	0.039
41	228	14.1	10.3		0.033	42	164	13.8	9.6		0.037

whether obtained from the diet or stored in liver and spleen, is utilized for the production of hemoglobin when copper is fed.

The finding of Rose and Kung ('32), that more hemoglobin had to be withdrawn to make a female anemic than a male gave strong presumptive evidence of the hemoglobin formed, but the data of this study do not furnish a final answer to the question. An estimate of the total blood in the body of each rat was possible, using the formula of Chisholm revised by Hortar as cited by Donaldson ('24). Such an estimate showed that the females of each series had more hemoglobin per gram of rat than the males of their respective groups. This difference amounted to 11% for series A and 14% for series B, thus paralleling fairly closely the differences in iron content, 13 and 12%.

It is interesting to note in this connection that the influence of sex on the storage of body constituents other than iron has been reported in the literature for both calcium and vitamin A. Sherman and MacLeod ('25) reported that at all ages the percentage of calcium in females which had not reared young is higher than that in males of the same age receiving the same diet. Ender ('34), studying the vitamin A content of livers of bullocks and cows, concluded that there was four to five times as much of this vitamin in the liver of the female as in that of the male.

SUMMARY

The iron content of carefully matched young male and female rats depleted to approximately 4 gm. of hemoglobin per 100 cc. of blood had an iron content of 0.013 ± 0.0004 mg. of iron per gram of body weight.

The bodies of a group similarly depleted and then fed 0.0019 mg. of iron per gram of body weight until the hemoglobin reached 14 gm. per 100 cc. averaged for the males 0.030 ± 0.0003 mg. and for the females 0.034 ± 0.0005 mg. Another group fed 0.0038 mg. of iron per gram of body weight averaged for males 0.034 ± 0.0006 mg. and for females

0.038 ± 0.0007 mg. A third group fed 0.0057 mg. of iron per gram of body weight averaged 0.033 mg. for males and 0.037 mg. for females. The females stored on the average about 12% more iron than the males when the intake was the same per gram of body weight.

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